



REC'D	16 FEB 2005
WIPO	PCT

**Intyg  
Certificate**

Härmed intygas att bifogade kopior överensstämmer med de handlingar som ursprungligen ingivits till Patent- och registreringsverket i nedannämnda ansökan.

This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.

(71) Sökande              Gyros AB, Uppsala SE  
Applicant (s)

(21) Patentansökningsnummer    0400181-4  
Patent application number

(86) Ingivningsdatum              2004-01-29  
Date of filing

Stockholm, 2005-02-08

För Patent- och registreringsverket  
For the Patent- and Registration Office

Gunilla Larsson

Avgift  
Fee

**PRIORITY  
DOCUMENT**

SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH RULE 17.1(a) OR (b)

GY 0068SE

1

2004-0129

Huvudfaxen Kassan

**SEGMENTED POROUS BEDS AND PRELOADED MICROSCALE DEVICES****TECHNICAL FIELD**

The invention relates to a microfluidic device for performing experiments which each 5 comprises interaction between a solid phase material and a solute that is present in a liquid. The solid phase material is present in the device as porous beds during the experiments. The device permits that one or more experiments can be carried out in parallel.

- 10 In the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> aspects an important feature is that the porous bed is in a dry state and comprises a bed-preserving agent. See claims 4-10. In the fourth aspect an important feature is that the porous bed comprises an upstream part and a downstream part where a reactant that is capable of reacting with a solute in a through-passing liquid primarily is immobilized to the solid phase material of the downstream part.
- 15 See claims 1-3.

Parallelity means that at least a sequence containing the binding step is similar for the experiments, i.e. the periods of time during which the solid phase material is in contact with the corresponding solutions and the sequence in which the solutions are 20 used are essentially the same. The reagents/reactants used may be different.

The term "solute" comprises true solutes, microorganisms including viruses, suspended cells, suspended cell parts and various other reactants that are in dissolved or colloidal form and sufficiently small to be transported by liquid flow through the 25 bed.

The term "microfluidic device" means that the device comprises one or more microchannel structures in which liquid flow is used for transporting various kinds of reactants, analytes, products, samples, buffers and/or the like. The terms "micro" in 30 "microchannel structure" contemplates that there are one or more cavities and/or conduits that have a cross-sectional dimension that is  $\leq 10^3 \mu\text{m}$ , preferably  $\leq 5 \times 10^2 \mu\text{m}$ , such as  $\leq 10^2 \mu\text{m}$ . The device is capable of processing liquid aliquots in the nanolitre (nl) range (which includes the picolitre (pl) range). The nl-range has an

29-01-2004 11:28

FRAN-GYROS AB

+46 18 566350

+46 18 566350

T-025 P.007 F-406  
Ink. t. Patent- och reg.verket

SE 01-29

GY 0068SE

2

Huvudtaxen Kassan

2004-0129

upper end of 5,000 nl but relates in most cases to volumes  $\leq$  1,000 nl, such as  $\leq$  500 nl

or  $\leq$  100 nl.

The interaction between the solute and the porous bed contemplates e.g.

- 5 a) separation of the solute from the liquid, i.e. the solute is retained by the solid so that bed and the solute can be separated from each other,  
b) interaction as part of a catalytic reaction, e.g. an enzymatic reaction, and/or  
c) solid phase synthesis.

10 Patent publications cited herein are incorporated by reference in their entirety.

#### BACKGROUND PUBLICATIONS

WO 0275312 (Gyros AB) focuses on affinity assays for the characterization of reaction variables by binding a soluble affinity reactant to a solid phase material that 15 comprises in immobilized form the counterpart to the affinity reactant. The solid phase is represented by the inner wall of the reaction microcavity or by a porous bed placed in the reaction microcavity.

SE 0201310 (Gyros AB) describes performing catalytic assays with one part of the 20 used catalytic system being immobilized. The assays are illustrated with enzyme systems. The immobilization techniques and support materials are in principle the same as in WO 0275312 (Gyros AB).

US 5,726,026 (Univ. Pennsylvania) and US 5,928,880 (Univ. Pennsylvania) describe 25 in a side sentence a microfluidic device that comprises a detection/reaction zone containing a solid phase material in particle form. Streptavidin is immobilized to the particles. The particles may be dried or lyophilized.

US 6,479,299 (Caliper) discusses predispensation of soluble and insoluble reagents 30 (assay components) during the manufacture of a microfluidic device. Insoluble reagents may be in lyophilized form.

+4618566350

GY 0068SE

3

2004-0129

Applicant has marketed a microscale fluidic device (Gyrolab MALDI SP1) containing a plurality of microchannel structures each of which contains a column of a reverse solid phase material (hydrophobic beads) (WO 02075775 (Gyros AB) and WO 0275776 (Gyros AB)). The solid phase material is in a dry state. In order to secure

Ink 1 Patent- och reg.verket  
R 111 -01- 2 9

Huvudlinjen Kassan

- 5 that the beads are retained in the correct location during storage and transport, the packages of the devices have been specifically designed.

WO 56808 (Gyros AB), WO 0147437 (Gyros AB), WO 0154810 (Gyros AB), WO 02075775 (Gyros AB) and WO 0275776 (Gyros AB) suggest in general terms to

10 deliver microfluidic devices in dry form.

US 5,354,654 (Ligler et al) suggests a kit comprising a solid support with an immobilized ligand-receptor complex that has been lyophilized together with a cryostabilizer. Packing of the support in a macroscale column is suggested.

15

US 5,998,155 (Squibb) and US 5,691,152 (Squibb) describes compositions having a high biotin-binding activity and comprising a biotin-binding moiety immobilized to a polymer support. The support may be in beaded form and lyophilized together with (a) a bulking agent protecting the beads from damages during freeze-drying and

20 assisting the reswelling of the beads, (b) a protectant for inhibiting chemical reactions during freeze-drying and storage, (c) buffers etc.

#### BACKGROUND PROBLEMS

There are a number of technical problems associated with providing the market with

25 microfluidic devices of the type discussed above. We have found that in the case the customer would introduce the hydrophilic porous bed into the device, there will be a high risk for obtaining mal-functioning beds. In total this would lead to increased inter- and intra-device variations in performance of the beds/microchannel structures, decreased sensitivity and reproducibility, etc.

30

In the macroworld the general trend has been to provide preloaded columns with solid phase based separation media in bed form in a wet state and easily controlled. Losses of liquid during storage due to evaporation typically are low compared to the total

GY 0068SE

4

-01- 2 g

2004-0129

volume. The situation is quite different for microfluidic devices where bed volumes typically are in the nl-range. Evaporation is assisted by wicking, easily becomes significant and results in quick uncontrolled drying of a bed and an unacceptable risk for the creation of channels, cavities and inclusion of air that will disturb the liquid

- 5 flow characteristics of the bed. For solid phase material comprising a bioactive reactant the risk for irreproducible and irreversible changes in activity is also apparent. There are difficulties in reconstituting fully or partly dried solid phase material in microfluidic devices to minute well-ordered and homogeneous porous beds/columns having the liquid flow characteristics and binding activity with  
10 essentially the same inter-channel and inter-device variation as the wet beds had before drying.

These problems are typically more pronounced for hydrophilic and/or water-swellable solid phase material than for hydrophobic that do not swell in water. See figures 2a-b  
15 and 3.

- Our experience with wet hydrophilic beds implanted the idea that the beds have to be dried under controlled conditions. It still, however, turned out difficult to implement dried solid phase material that could be reconstituted in the desired way to minute  
20 porous beds/columns, e.g.
- The solid phase material typically carries a reactant that is sensitive to drying, storage and transportation.
  - The binding of the solute to a porous bed in a microfluidic device may in many variants of microfluidic devices be monitored by spectrometric methods through a  
25 detection window associated with the porous bed. The creation of undesired channels, cavities and air inclusions will increase the noise level for detection and thus also reduce sensitivity and reproducibility.
  - During transportation of microfluidic devices that comprises porous beds, there is a significant risk that solid phase material may escape from the microcavity in  
30 which it is originally placed. The risk for losses of dispensed reagents and analyte by reactions with escaped solid phase material at undefined locations within a microchannel structure is apparent. This kind of problem is most severe if the bed is built up of particles.

GY 0068SE  
2004-01-29

5

2004-01-29

Huvudforsen Kassan

**OBJECTS OF THE INVENTION**

The objects are to provide improved microfluidic devices that solve the problems discussed above. The objects thus comprise to provide microfluidic devices

- 5 comprising solid phase material in a dry state that after storage and transportation of the device can be reconstituted to wet beds with essentially the same performance as wet beds of the same solid phase material not having been transformed to the dry state. If the solid phase material comprises an immobilized reactant, its activity, e.g. binding activity such as capacity, shall be essentially unchanged by transformation to
- 10 the dry state, storage, transportation and reconstitution. This in particular applies to activity under flow conditions. The objects include providing methods for manufacturing the devices and use of the devices for separation and/or assay purposes, among others.

**15 THE INVENTION**

It has now been discovered that there are certain compounds and/or combinations of compounds that when intimately mixed with a solid phase material will reduce adverse effects of predisposing, drying, storage, transportation, reconstitution etc of solid phase materials intended to be used as minute porous beds in microfluidic

- 20 devices. These negative effects are for example:
  - a) unacceptable formation of channels, cavities, air inclusions etc and/or,
  - b) escape of solid phase material from a desired location within a microchannel structure, and/or
  - c) reduction of the binding activity of an immobilized reactant, e.g. affinity

25 reactant  
This kind of compound or combination of compounds will henceforth be called "bed-preserving agent" or simply "preserver" since they will assist in restoring a dried solid phase material to an efficient wet porous bed. A bed-preserving agent is simply included in the liquid phase of a wet solid phase material before drying/dehydration.

- 30 Drying can take place inside or outside the microfluidic device. By using the proper microfluidic distribution manifold, we have found that the accuracy for the formation of reconstituted wet beds of predetermined volume can be further increased in each microchannel structures/reaction microcavity connected to the manifold. Inter-channel

29-01-2004

11:28

FRAN-GYROS AB

+46 18 566350

+46 18 566350

T-025

P.011

F-406

Ink. t. Patent- och reg.verket

GY 0068SE

6

2004-0129

Huvudfoxen Kassan

variations due to drying, storage, transportation and/or reconstitution of preloaded solid phase materials can easily be held at a minimum.

- It has also been discovered that common flow control as defined in WO 0275312 is
- 5 beneficial for increasing the accuracy in restoring wet porous bed volumes in parallel in reaction microcavities of at least a subset of microchannel structures of a microfluidic device. Centrifugal force, for instance, is useful for improving the yield of efficient porous beds if applied for settling and restoring the beds.

10 **FIRST ASPECT: MICROFLUIDIC DEVICE**

- This aspect is a microfluidic device that comprises one, two or more microchannel structures, each of which comprises a reaction microcavity intended for retaining a solid phase material in the form of a porous bed. The device is characterized in that each microchannel structure comprises a reaction microcavity with a hydrophilic solid phase material in a dry state that comprises one or more compounds that act as bed-preserving agents and secure that an acceptable wet porous bed is restored after a reconstitution liquid has passed the dry state solid phase material. The bed preserving agent(s) are capable of
- a) stabilizing the solid phase material possibly containing an immobilized reactant
- 20 (e.g. an affinity reactant) during
- (i) transformation of a wet state of the solid phase material to a dry state, and/or
- (ii) a subsequent storage and/or transportation, and/or
- b) assisting in the reconstitution of the dry state to a wet porous bed.

- 25 The term "acceptable wet porous bed" means that the experimental results from the bed can be used, i.e. the bed is functional. The term "unacceptable" means that the experimental results are discarded. The bed-preserving agent used thus increases the probability for obtaining acceptable beds. The use of the principles of the invention may thus assist in increasing the yield of functional beds/microchannel structure on a
- 30 microfluidic device to become  $\geq 70\%$ , such  $\geq 80\%$  or  $\geq 90\%$  or  $\geq 95\%$  or  $\geq 98\%$  of the total number of beds of a microfluidic device.

29-01-2004 11:29

FRAN-GYROS AB

+46 18 566350

+46 18 566350

T-025

P.012

F-406

Ink. t. Patent- och reg.verket

GY 0068SE

7

2004-01-29

Huvudtakson Kassan

By the term "dry state" is meant that the amount of remaining liquid after drying is  $\leq$  50 %, such as  $\leq$  30 % or  $\leq$  20 % or  $\geq$  10 % of the amount of liquid present in the solid phase material when saturated with the liquid concerned (with no free liquid layer appearing on top of the bed). In many cases this means that the amount of liquid in the solid phase material after drying and/or storage is  $\leq$  20 % (w/w), such as  $\leq$  10 % or  $\leq$  5 %. The liquid referred to is typically water.

#### Bed-preserving agents (additives)

- The damages of a porous bed during drying/dehydration and storage typically depend
- 10 on stresses induced during transformation from a wet state to a dry state in the similar manner as for biologically active material. The choice of bed-preserving agent will depend on the conditions for drying, the solid phase material, kind of immobilized reactant etc. The same compound(s) may act as bed-preserving agent for one solid phase material and/or immobilized reactant but negatively affect other combinations.
- 15 It will thus be extremely important to test individual preserver candidates [either as single compounds or as combination(s) of compounds] and conditions for the transformation to the dry state and/or the conditions for storage and/or reconstitution before a candidate is used for a particular solid phase material. Testing is typically by trial and error and may include
- 20 a) physical inspection of the bed to find undesired channels, cavities and air inclusions, and/or  
b) determination of through flow properties, activity of an immobilized reactant, etc.  
Determination of the activity of the immobilized reactant/ligand may include determination of i) the activity profile in the flow direction and/or perpendicular to the
- 25 flow direction (i.e. the distribution of activity in the bed), ii) the total activity of the bed etc, for instance by testing the bed behavior in a standard type of assay or in an actual future use of the porous bed. If the immobilized reactant is an affinity reactant that is able to capture a solute, the distribution of the solute in the bed after adsorption may be used to find abnormal local behavior caused by channels, cavities, air
- 30 inclusions or local inactivation of the reactant, for instance. The total amount of adsorbed solute may give a total view, e.g. a measure of the mean condition of the immobilized reactant after reconstitution. Adsorption in this context is preferably performed under flow conditions, i.e. a liquid containing the solute is allowed to flow

GY 0068SE  
2004-0129

8

Ink t. Patent- och reg.verket

VIA -01- 2 9

Huvudlagen Kassan

through the porous bed. These kinds of testing typically include a comparison with a standard bed and/or standard behavior that may be given by

- a) tabulated values,
- b) preset specifications,
- 5 c) the behavior of a bed prepared from non-lyophilized/non-dried solid phase material of the same kind as the lyophilized/dried solid phase material to be tested, etc.

The substeps during which the risk for damages is most significant are primarily the

- 10 drying step (dehydration step) and the storage as such. In the case freeze-drying is part of the transformation also the freezing step may cause significant damages. For biologically active material, it is well known that particular stabilisators may be required for each substep. Hence, cryostabilisators refer to freezing, lyostabilisators to dehydration/drying and long term stabilisators to storage. See for instance Arakawa et 15 al (Advanced Drug Delivery Reviews 46 (2001) 307-326). In the context of the present invention the analogous categorization is used for bed-preserving agents.

Compounds that assist in the reconstitution of the dry solid phase material to the wet porous bed are called bed-reconstitution agents and are also bed-preserving agents.

20

A bed-preserving agent may be active in relation to at least one up to all of the steps: drying/dehydration, freezing, storage and reconstitution. The efficiency of a particular agent will depend on the conditions for the particular step, solid phase material and/or immobilized reactant to be stabilized.

25

A bed-preserving agent that is useful in the present invention typically is hydrophilic in the sense that it is water-soluble. Many bed-preserving agents thus exhibit one or more heteroatoms selected from oxygen, nitrogen and sulphur, typically with a ratio between the total number of carbon atoms and the total number of oxygen, nitrogen

30 and sulphur atoms which is  $\leq 6$ , such as  $\leq 4$  or  $\leq 2$ .

Typical bed-preserving agents may be found in the group consisting of compounds exhibiting a) carbohydrate structure which also includes sugar alcohol structure, b)

GY 0068SE

9

2004-0129

polyhydroxy structure (i.e. organic polyols which also includes polyhydroxy polymers), c) amino acid structure including peptide structure and imino acid structure, d) inorganic salts, e) organic salts in particular carboxylates, f) amine structure including amino acid structure and ammonium structure, h) etc.

5

Suitable compounds with carbohydrate structures may be found amongst sucrose, lactose, glucose, trehalose, maltose, isomaltose, cellobiose, inositol, ethylene glycol, glycerol, sorbitol, xylitol, mannitol, polyethylene glycol possibly substituted in one or both of its end, dextran, maltodextrin, monosaccharides, disaccharides,

10 polysaccharides including oligosaccharides etc. Compounds with carbohydrate structures are typically also polyols.

Suitable polyols may be found amongst polyhydroxy polymers, such as polysaccharides, polyvinylalcohol possibly partially substituted on its hydroxy groups

15 for instance with acetate or lower hydroxy alkyl groups ( $C_{2-4}$ ), poly (lower hydroxy alkyl ( $C_{2-4}$ ) acrylate) polymers and corresponding poly methacrylate polymers etc, and monomeric compounds having two or more hydroxy groups. In a typical polyol each hydroxy group is attached directly to an  $sp^3$ -hybridised carbon.

20 Suitable polymers are typically found amongst polymers that have a plurality of functional groups comprising a heteroatom selected from oxygen and nitrogen. Relevant functional groups are  $-O(CH_2CH_2O)_n-$  where n is  $\geq 2$  such as  $\geq 5$ , amido such as  $-CONH-$  or  $-CONH_2$  where H may be replaced with a suitable hydrophilic organic group, hydroxy (OH), ester ( $-COOR$ , where R is a suitable hydrophilic

25 organic group), etc. Specific examples are polyethylene glycol, dextran and other polysaccharides, polyvinylpyrrolidone, polypeptides, the poly acrylate and methacrylate polymers mentioned above, the polyvinyl alcohols mentioned above etc.

In the bed-preserving candidates mentioned above the term polymer also includes  
30 copolymer in which the specific polymer structure mentioned is a part

Bed-preserving agents that are lyostabilisators are believed to act during the drying/dehydration step by replacing water bound to the solid phase material to be

GY 0068SE  
2004-0129

10

stabilized. These bed-preserving agents thus primarily are found among compounds that may participate in hydrogen bonding/coordination with the solid phase material.

With the present knowledge the most typical candidates for lyostabilization are found amongst polyols (including diols, triols etc), e.g. with a polymeric structure and/or

5 carbohydrate structure (oligomeric is included in polymeric). In the case the solid phase material comprises an immobilised reactant, e.g. with peptide structure, it is believed that the most efficient candidates have carbohydrate structure with preference for disaccharides and found amongst sucrose, lactose, glucose, trehalose, maltose, isomaltose, cellobiose etc.

10

Many times suitable bed-preserving agents, such as lyostabilisators and stabilisators for long term storage, may exist in a glassy state at an ordinary temperature. An ordinary temperature in this context means at least a part of the interval + 15-35°C.

Typically this means a glass transition temperature  $\geq + 25^{\circ}\text{C}$  or  $\geq + 30^{\circ}\text{C}$  or  $\geq + 35^{\circ}\text{C}$ .

15

The bed-preserving agents that are present in the dry state of a solid phase material are typically non-volatile. This does not exclude having volatile cryostabilisators included during lyophilization.

## 20 Protectants (additives)

The solid phase material that is in a dry state may also contain one or more so-called protectants that inhibit undesired chemical reactions of the solid phase material and/or the immobilized reactant. Suitable protectants are found amongst free radical scavengers, antioxidants, reducing agents etc.

25

## Other additives

The solid phase material in a dry state may also contain an appropriate buffer, such as a buffer with non-volatile buffering components, e.g. with at least one or two of the buffering components being anionic, such as in phosphate buffers, citrate buffers etc.

30

Also other buffers may be used. The buffering components typically provide an elevated buffer capacity within an appropriate pH interval of the range of pH 1-13 with preference for the range 3-11. For lyophilized solid phase materials, phosphate buffers, in particular with potassium as counter-ion, are preferred.

Huvudläxen Kassan

JULI -01- 2 9

29-01-2004 11:29

FRAN-GYROS AB

+46 18 566350

+46 18 566350

T-025 P.016

F-406

GY 0068SE  
2004-0129

11

Ink. t. Patent- och reg.verket

2004-01-29

Huvudfaxon Kassan

Other additives such as one or more antimicrobial agents may also be included, e.g. a bacteriostat, a bacteriocid, a virucid etc.

5 A possible bulking agent may also be included as an additive. The bulking agent may have bed-preserving effects on the solid phase material as discussed above for bed preserving agents in general.

10 Microcavity adherence agents (a kind of bed-preserving agents) cause the solid phase material to be retained in a reaction microcavity and therefore assist in restoring a dry state solid phase material to a wet porous bed. This kind of agents acts by causing particles to adhere to each other and/or to the inner walls of a reaction microcavity. Microcavity adherence agents may be found amongst the bed-preserving candidates discussed above, for instance amongst those that exhibit carbohydrate and/or 15 polymeric structure.

20 The various additives (bed preserving agents, buffer substances, protectants, bulking agents etc) are typically present in the solid phase material that is in the dry state in an amount in the interval of 0.001 - 25 %, such as  $\geq 0.01\%$  or  $\geq 0.1\%$  and/or  $\leq 10\%$  or  $\leq 1\%$ . These intervals apply to each individual additive as well as to the total amount of additive with the proviso that the total amount should not exceed the upper limit of an interval. The determination of optimal ranges of efficient amounts and sufficient bed-preserving effects of individual bed-preserving agents needs experimental testing as discussed above. The %-figures refer to the weight of the additive(s) relative to the 25 total weight of solid phase material in the dry state.

30 Additives (stabilisators, buffer substances, protectants, antimicrobials and/or bulking agents) are typically soluble in aqueous media so that they easily can be removed from the reconstituted porous bed.

#### **Reaction microcavity and the solid phase material.**

The reaction microcavity is defined as the part of a microchannel structure in which the solid phase is present. This means that for solid phases in the form of porous beds,

GY 0068SE  
2004-0129

12

2004-01-29

the bed volume and the reaction microcavity will coincide and have the same volume<sup>and foxen Kassan</sup>  
If the solid phase is the inner wall of a microconduit, the reaction microcavity is  
defined as the volume between the most upstream and the most downstream end of  
the solid phase.

5

The reaction microcavity is typically a straight or bent microconduit that may or may  
not be continuously widening and/or narrowing. On the same device all reaction  
microcavities typically have essentially the same shape and/or size. In a microfluidic  
device that comprises reaction microcavities according to the invention that differ in  
10 shape and/or size, the reaction microcavities/microchannel structures may be divided  
into groups where each group contains reaction microcavities that are not present in  
any of the other groups. Each group may be placed in a subarea of the device that is  
separate from the subareas of other groups.

15 The reaction microcavity has at least one cross-sectional dimension that is  $\leq$  1,000  
 $\mu\text{m}$ , such as  $\leq$  500  $\mu\text{m}$  or  $\leq$  200  $\mu\text{m}$  (depth and/or width). The smallest cross-sectional  
dimension is typically  $\geq$  5  $\mu\text{m}$  such as  $\geq$  25  $\mu\text{m}$  or  $\geq$  50  $\mu\text{m}$ . The total volume of the  
reaction microcavity is typically in the nl-range, such as  $\leq$  5,000 nl, such as 1,000 nl  
or  $\leq$  500 nl  $\leq$  100 nl or  $\leq$  50 nl or  $\leq$  25 nl.

20

The porous bed is a) a population of porous or non-porous particles, or b) a porous  
monolith.

A monolithic bed may be in the form of a porous membrane or a porous plug.

25

The term "porous particles" have the same meaning as in WO 0275312 (Gyros AB).

30

Suitable particles are spherical or spheroid (beaded) or non-spherical. Suitable mean  
diameters for particles used as solid phases are typically found in the interval of 1-100  
 $\mu\text{m}$  with preference for mean diameters that are  $\geq$  5  $\mu\text{m}$ , such as  $\geq$  10  $\mu\text{m}$  or  $\geq$  15  $\mu\text{m}$   
and/or  $\leq$  50  $\mu\text{m}$ . Also smaller particles can be used, for instance with mean diameters  
down to 0.1  $\mu\text{m}$ . The outlet of the reaction microcavity and the particles should match

GY 0068SE  
2004-0129

13

2004-01-29

each other so that the particles can be retained in the reaction microcavity. Certain kinds of particles, in particular particles of colloidal dimension, may agglomerate. In these cases the size of the agglomerate should be in the intervals given even if the agglomerating particles as such are below. See for instance WO 0275312 (Gyros AB).

5 Diameters refer to the "hydrodynamic" diameters.

Particles to be used may be monodisperse (monosized) or polydisperse (polysized) in the same meaning as in WO 0275312 (Gyros AB).

10 The solid phase material may or may not be transparent.

The base material of a solid phase may be made of inorganic and/or organic material. Typical inorganic materials comprise glass and typical organic materials comprise organic polymers. Polymeric materials comprise inorganic polymers, such as glass,

15 and organic polymers that may be of synthetic or biological origin (biopolymers). The term biopolymer includes semi-synthetic polymers in which there is a polymer backbone derived from a native biopolymer. Typical synthetic organic polymers are cross-linked and are often obtained by the polymerisation of monomers comprising polymerisable carbon-carbon double bonds. Examples of suitable monomers are

20 hydroxy alkyl acrylates and corresponding methacrylates, acryl amides and methacrylamides, vinyl and styrol ethers, alkene substituted polyhydroxy polymers, styrene, etc. Typical biopolymers may or may not be cross-linked. In most cases they exhibit a carbohydrate structure, e.g. agarose, dextran, starch etc.

25 The term "hydrophilic" in the context of a porous bed contemplates ability to absorb water when contacted with water. The expression also means that the inner surfaces of the bed that is in contact with water during the absorption shall expose a plurality of polar functional groups which each has a heteroatom selected amongst oxygen and nitrogen, for instance. Appropriate functional groups can be selected amongst

30 hydroxy groups, ethylene oxide groups ( $-X-[CH_2CH_2O]_n$ , where n is an integer > 1 and X is nitrogen or oxygen), amino groups, amide groups, ester groups, carboxy groups, sulphone groups etc, with preference for those groups that are essentially uncharged independent of pH, for instance within the interval of 2-12. For solid phase

GY 0068SE

14

1001-2 g

2004-0129

Frövallan Kassan

materials in particle form this means that at least the outer surfaces of the particles have to exhibit polar functional groups. The hydrophilic functional groups may be present on or be a part of so called extender arms (tentacles).

- 5 If the base material of a solid phase material is hydrophobic or not sufficiently hydrophilic, e.g. is based on a styrene (co)polymer, the surfaces that are to be in contact with an aqueous liquid may be hydrophilized. Typical protocols comprise coating with a compound or mixture of compounds exhibiting polar functional groups of the same type as discussed above, treatment by an oxygen plasma etc.

10

The solid phase material may be swellable or not swellable in water or other aqueous liquid media. Swellable materials are likely to be more prone to give problem related to (a) shrinkage, and inhomogeneous packing and/or through flow after reconstitution and/or (b) escape of dry particles during storage and transportation. The term

- 15 "swellable" in this context means that an increase in volume of the material (particles as such or a monolith) can be detected or is  $\geq 10$  or  $\geq 75\%$  when the material in the dry state (as defined above) is contacted with excess water. The temperature referred to is typically +25°C.

- 20 The solid phase material may be rigid or elastic.

The solid phase material may or may not contain an immobilized reactant that is capable of participating in an organic, an inorganic, a biochemical reaction etc. Depending on the circumstances and the kind of reactant and the solutes, the

- 25 interaction may be part of a separation process, a catalytic reaction, a solid phase synthesis etc.

- This reactant will now be illustrated with an affinity reactant that is an affinity counterpart ( $AC_S$ ) to a solute ( $S$ ) and capable of forming an affinity complex ( $AC_S-S$ ) with the solute. Affinity bonds typically are based on: (a) electrostatic interactions, (b) hydrophobic interactions, (c) electron-donor acceptor interactions, and/or (d) bioaffinity binding.

GY 0068SE  
2004-0129

15

2004-01-29

Bioaffinity binding typically is complex and comprises a combination of interactions.

Huvudfrågan Kassan

An immobilized affinity counterpart (ACs) may thus:

- (a) be electrically charged or chargeable, i.e. contains positively charged nitrogen
  - 5 (e.g. primary, secondary, tertiary or quaternary ammonium groups, and amidinium groups) and/or negatively charged groups (e.g. carboxylate groups, phosphate groups, phosphonate groups, sulphate groups and sulphonate groups); and/or
  - (b) comprise one or more hydrocarbyl groups and other hydrophobic groups; and/or
  - (c) comprise one or more heteroatoms (O,S,N), possibly linked to hydrogen and/or
- 10 (d) comprise a combination of features (a)-(c).

- A bioaffinity reactant/ligand is a member of a bioaffinity pair. Typical bioaffinity pairs are a) antigen/hapten and an antibody, b) complementary nucleic acids, c)
- 15 immunoglobulin-binding protein and immunoglobulin (for instance IgG or an Fc-part thereof and protein A or G), d) lectin and the corresponding carbohydrate, e) biotin and (strept)avidin, e) members of an enzymatic system (enzyme-substrate, enzyme-cofactor, enzyme-inhibitor etc), f) an IMAC group and an amino acid sequence containing histidyl and/or cysteinyl and/or phosphorylated residues (i.e. an IMAC motif), etc. Antibody includes antigen binding fragments and mimetics of antibodies. The term "bioaffinity pair" includes also affinity pairs in which one or both of the members are synthetic, for instance mimicking one or both of the members of a native bioaffinity pair. The term IMAC stands for an immobilized metal chelate.
  - 25 The term "affinity reactant" also includes a reactant that is capable of reversible covalent binding, for instance by disulfide formation. This kind of reactants typically exhibits a HS- or a -S-SO<sub>n</sub>- group (n = 0,1 or 2, free valences bind to carbon). See US 5,887,997 (Batista), US 4,175,073 (Axén et al), and 4,563,304 (Axén et al).
  - 30 The immobilized reactant/ligand (affinity reactant) may also be a catalytic system or a member of a catalytic system, such as a catalyst, a cocatalyst, a cofactor, a substrate or cosubstrate, an inhibitor, a promotor etc. For enzymatic systems the corresponding members are enzyme, cocatalyst, cofactor, coenzyme, substrate, cosubstrate etc. The

GY 0068SE  
2004-0129

16

1124-01-29

Hans-Joachim Kassahn

term "catalytic system" also includes linked catalytic systems, for instance a series of systems in which the product of the first system is the substrate of the second catalytic system etc and whole biological cells or part of such cells.

- 5 The immobilized affinity reactant (AC<sub>s</sub>) should be selected to have the appropriate selectivity and specificity for binding the solute to the solid phase material in relation to an intended application. General methods and criteria for the proper selection of affinity reactants and reaction conditions are well known in the field.
- 10 The affinity constant ( $K_{S-AC} = [S][AC_s]/[S-AC_s]$ ) for the formation of the complex comprising the immobilized affinity reactant (AC<sub>s</sub>) and the solute (S) is an important criterion for optimizing an application and varies depending on application. For affinity assays the affinity constant is typically  $\leq 10^{-8}$  mole/l or  $\leq 10^{-9}$  mole/l. This kind of assays typically includes that the solute is reacted with immobilized AC<sub>s</sub>
- 15 under flow conditions and related to the amount of an analyte in an animal or biological sample (animal or biological sample include samples from mammals, such as human and other animal patients, and from experimental animals). This does not exclude that affinity counterparts having weaker affinities may be used for this kind of samples, other samples and affinity assays, and other applications. Thus depending on
- 20 application the affinity constant may be relatively large, such as up to  $10^{-3}$  mole/l or up to  $10^{-4}$  mole/l or up to  $10^{-5}$  or up to  $10^{-7}$  mole/l, or relatively low, such as less than  $10^{-8}$  mole/l or less than  $10^{-11}$  mole/l.

The techniques for immobilization of a reactant/ligand may be selected amongst

- 25 techniques that are commonly known in the field. The linkage to the solid phase material may thus be via covalent bonds, affinity bonds (for instance biospecific affinity bonds), physical adsorption etc.

Immobilization via affinity bonds may utilize an immobilizing affinity pair in which

- 30 one of the members (immobilized ligand or L) is firmly attached to the solid phase material, for instance covalently. The other member (immobilizing binder, B) of the pair is used as a conjugate (immobilizing conjugate) comprising binder B and the affinity counterpart AC<sub>s</sub> to the solute S. Examples of immobilizing affinity pairs are

GY 0068SE

17

J-01-2 g

2004-0129

Huvudlinjen Kassan

a) streptavidin/avidin/ neutravidin and a biotinylated reactant (or vice versa), b) antibody and a hapteneated reactant (or vice versa), c) an IMAC group and an amino acid sequence containing histidyl and/or cysteinyl and/or phosphorylated residues (i.e. an IMAC motif) linked to a reactant, etc.

5

The term "conjugate" primarily refers to covalent conjugates, such as chemical conjugates and recombinantly produced conjugates (where both the moieties have peptide structure). The term also includes so-called native conjugates, i.e. affinity reactants exhibiting two binding sites that are spaced apart from each other, with 10 affinity directed towards two different molecular entities, for instance a native antibody that comprises species and class-specific determinants on one side of the molecule and antigen/hapten-binding sites on another side.

It is believed that it is advantageous that the immobilized ligand L has two or more 15 binding sites for the immobilizing binder B, and/or the immobilizing binder B has one, two or more binding sites for the ligand L (or vice versa).

Preferred immobilizing affinity pairs (L and B) typically have affinity constants ( $K_{L-B} = [L][B]/[L-B]$ ) that are at most equal to or  $\leq 10$  times or  $10^2$  times or  $\leq 10^3$  times 20 larger than the corresponding affinity constant for streptavidin and biotin. This typically will mean affinity constants that roughly are  $\leq 10^{-13}$  mole/l,  $\leq 10^{-12}$  mole/l,  $\leq 10^{-11}$  mole/l and  $\leq 10^{-10}$  mole/l, respectively. The preference is to select L and B amongst biotin-binding compounds and streptavidin-binding compounds, respectively, or vice versa.

25

The affinity constants discussed above refer to values obtained by a biosensor (surface plasmon resonance) from Biacore (Uppsala, Sweden), i.e. with the affinity reactant (AC<sub>S</sub> and L) immobilized to a dextran-coated gold surface.

30 At least one of the members of an affinity pair, in particular a bioaffinity pair, to be used in the present invention typically exhibits a structure selected amongst: a) amino acid structure including peptide structure such as poly and oligopeptide structure, b) carbohydrate structure, c) nucleotide structure including nucleic acid structure, d)

GY 0068SE  
2004-0129

18

Hans-Henrik Kassan

lipid structure such as steroid structure, triglyceride structure etc. The term affinity pair in this context refers to the immobilizing affinity pair (L and B), the immobilized affinity reactant and the solute (AC<sub>S</sub> and S) and other affinity pairs that may be used.

- 5 The solid phase material that is in a dry state may alternatively be in activated form. In other words ready for direct covalent immobilization by reaction with a functional group of a desired reactant. The functional group that can be used on the desired reactant is typically selected amongst electrophilic and nucleophilic groups and depends on whether or not the activated group is nucleophilic or electrophilic.
- 10 respectively. Examples of functional groups that may be used are amino groups and other groups comprising substituted or unsubstituted -NH<sub>2</sub>, carboxy groups (-COOH/-COO<sup>-</sup>), hydroxy groups, thiol groups, keto groups etc.

#### Other features of the microfluidic device

- 15 A microchannel structure of the microfluidic device may comprise one, two, three or more functional parts selected among: a) inlet arrangement comprising for instance an inlet port/inlet opening, possibly together with a volume-metering unit, b) microconduits for liquid transport, c) reaction microcavity; d) mixing microcavity; e) unit for separating particulate matters from liquids (may be present in the inlet arrangement), f) unit for separating dissolved or suspended components in the sample from each other, for instance by capillary electrophoresis, chromatography and the like; g) detection microcavity; h) waste conduit/microcavity; i) valve; j) vent to ambient atmosphere; etc. A functional part may have more than functionality, e.g. reaction microcavity and a detection microcavity may coincide. Various kinds of
- 20 functional units in microfluidic devices have been described by Gyros AB/Amersham Pharmacia Biotech AB: WO 9955827, WO 9958245, WO 02074438, WO 0275312, PCT/SE02/01539, PCT/SE02/01701 and by Tecan/Gamera Biosciences: WO 0187487, WO 0187486, WO 0079285, WO 0078455, WO 0069560, WO 9807019, WO 9853311.

30

In advantageous forms a reaction microcavity intended for a hydrophilic porous bed is connected to one or more inlet arrangements (upstream direction), each of which comprises an inlet port and at least one volume-metering unit. In one advantageous

GY 0068SE  
2004-0129

19

variant, there is one separate inlet arrangement per microchannel structure and reaction microcavity intended to contain the solid phase material. In another advantageous variant, the inlet arrangement is common to all or a subset of microchannel structures and reaction microcavity intended to contain the solid phase

5 material and comprises a common inlet port and a distribution manifold with one volume-metering unit for each microchannel structure/reaction microcavity of the subset. In both variants, each of the volume-metering units in turn is communicating with downstream parts of its microchannel structure/reaction microcavity.

Microchannel structures linked together by a common inlet arrangement and/or

10 common distribution manifold define a group of the microchannel structures of the device.

Typical inlet arrangements have been presented in WO 0274438 (Gyros AB), WO 0275312 (Gyros AB), WO 0275775 (Gyros AB) and WO 0275776 (Gyros AB).

15

The microfluidic device may also comprise other common microchannels/micro conduits connecting different microchannel structures. Common channels including their various parts such as inlet ports, outlet ports, vents, etc., are considered part of each of the microchannel structures they are communicating with.

20

Common microchannels make it possible to construe microfluidic devices in which the microchannel structures form networks. See for instance US 6,479,299 (Caliper).

Each microchannel structure has at least one inlet opening for liquids and at least one 25 outlet opening for excess of air (vents) and possibly also for liquids.

The microfluidic device may also comprise microchannel structures that have no reaction microcavity for retaining a solid phase material according to the invention.

30 The microfluidic device contains a plurality of microchannel structures/device intended to contain the solid phase according to the invention. Plurality in this context means two, three or more microchannel structures and typically is  $\geq 10$ , e.g.  $\geq 25$  or  $\geq 90$  or  $\geq 180$  or  $\geq 270$  or  $\geq 360$ . As discussed above the microchannel structures of a

GY 0068SE

20

Hans-Joachim Kassner

2004-0129

device may be divided in groups, each of which may for instance be defined by the size and/or shape of the reaction microcavity, by a common microchannel, such as a common inlet arrangement or manifold etc. The number of microchannel structures in a group is typically in the interval 1-99 %, such as 5-50 % or 5-25 % or 10-50%, of 5 the total number of microchannel structures of the device. This typically means that each group typically comprises from 3-15 or 3-25 or 3-50 microchannel structures. Each group may be located to a particular area of the device.

Different principles may be utilized for transporting the liquid within the microfluidic 10 device/microchannel structures between two or more of the functional parts described above. Inertia force may be used, for instance by spinning the disc as discussed in the subsequent paragraph. Other forces are capillary forces, electrokinetic forces, non-electrokinetic forces such as capillary forces, hydrostatic pressure etc.

15 The microfluidic device typically is in the form of a disc. The preferred formats have an axis of symmetry ( $C_n$ ) that is perpendicular to the disc plane, where n is an integer  $\geq 2, 3, 4$  or  $5$ , preferably  $\infty$  ( $C_\infty$ ). In other words the disc may be rectangular, such as square, and other polygonal forms but is preferably circular. Once the proper disc format has been selected centrifugal force may be used for driving liquid flow.  
20 Spinning the device around a spin axis that typically is perpendicular or parallel to the disc plane may create the necessary centrifugal force. In the most obvious variants at the priority date, the spin axis coincides with the above-mentioned axis of symmetry.

For preferred centrifugal-based variants, each microchannel structure comprises one 25 upstream section that is at a shorter radial distance than a downstream section relative to a spin axis. The reaction microcavity intended for the porous bed is then at a radial position intermediary to the two sections.

If centrifugal force is used for the formation and/or reconstitution of a particle bed 30 and/or for driving liquid flow through the bed, the reaction microcavity is typically oriented with the flow direction radially outwards from the spin axis.

GY 0068SE  
2004-0129

21

Huvudtakst Rasson

The preferred devices are typically disc-shaped with sizes and forms similar to the conventional CD-format, e.g. sizes that are in the interval from 10% up to 300 % of the conventional CD-radii. The upper and/or lower sides of the disc may or may not be planar.

5

Microchannels/microcavities of a microfluidic devices may be manufactured from an essentially planar substrate surface that exhibits the channels/cavities in uncovered form that in a subsequent step are covered by another essentially planar substrate (lid). See WO 9116966 (Pharmacia Biotech AB) and WO 0154810 (Gyros AB). Both substrates are preferably fabricated from plastic material, e.g. plastic polymeric material.

The fouling activity and hydrophilicity of inner surfaces should be balanced in relation to the application. See for instance WO 0147637 (Gyros AB).

15

The terms "wettable" (hydrophilic) and "non-wettable" (hydrophobic) contemplate that a surface has a water contact angle  $\leq 90^\circ$  or  $\geq 90^\circ$ , respectively. In order to facilitate efficient transport of a liquid between different functional parts, inner surfaces of the individual parts should primarily be wettable, preferably with a contact angle  $\leq 60^\circ$  such as  $\leq 50^\circ$  or  $\leq 40^\circ$  or  $\leq 30^\circ$  or  $\leq 20^\circ$ . These wettability values apply for at least one, two, three or four of the inner side-walls of a microconduit. In case one or more of the side-walls have a higher water contact angle this can be compensated for by a lower water contact angle for the remaining side-wall(s). The wettability, in particular in inlet arrangements should be adapted such that an aqueous liquid will be able to fill up an intended microcavity by capillarity (self suction) once the liquid has started to enter the cavity. A hydrophilic inner surface in a microchannel structure may comprise one or more local hydrophobic surface breaks in a hydrophilic inner side-wall, for instance for introducing a passive valve, an anti-wicking means, a vent solely function as a vent to ambient atmosphere etc. See for instance WO 9958245 (Gyros AB) and WO 0274438 (Gyros AB).

29-01-2004

11:30

FRAN-GYROS AB

+46 18 586350

+46 18 586350

T-025

P.027

F-406

Ink t Patent- och reg.verket

GY 0068SE  
2004-0129

22

J 01- 2 9

Contact angles refer to values at the temperature of use, typically +25°C, are static and can be measured by the method illustrated in WO 0056808 (Gyros AB) and WO 0147637 (Gyros AB).

Huvudtalen Kassan

**5 SECOND ASPECT: METHOD FOR THE TRANSFORMATION OF A PLURALITY OF WET POROUS BEDS TO A DRY/DEHYDRATED STATE THAT POSSIBLY IS RECONSTITUTED TO A PLURALITY OF WET POROUS BEDS.**

This aspect is a method as the defined in the heading of this section. The method is characterized in comprising the steps of:

- 10      i) providing a microfluidic device comprising a plurality of microchannel structures each of which comprises a reaction microcavity containing a hydrophilic porous bed saturated with a liquid containing a bed-preserving agent,
- 15      ii) transforming the bed in each reaction microcavity to a solid phase material that is in a dry and/or dehydrated state,
- 15      iii) possibly reconstituting the solid phase material obtained in step ii) to the wet porous beds of the microchannel structures.

This aspect also concerns a method for reducing the inter-channel variation in a microfluidic device with respect to performance of reconstituted porous beds.

The solid phase material may or may not exhibit a reactant that can interact with a solute. Various characteristics are discussed below and elsewhere in this specification.

25 Step (iii) is preferably carried out under flow conditions, for instance with residence time and flow rates through the bed as discussed for the third aspect of the invention.

Porous particle beds can be created by flowing a dispersion of particles through all or one or more subsets of the reaction microcavities of the microfluidic device. The 30 particles will then settle and form a porous bed at the outlet end of each microcavity. Bed formation may be facilitated by the use of gravity and/or the use of centrifugal force, the latter preferably acting along the flow direction of each reaction microcavity. The desired additives as discussed above are present in the liquid

GY 0068SE

23

2004-01-29

2004-0129  
dispersion and/or introduced by passing a liquid containing the additives through the bed after it has been formed. The microfluidic device together with the beds saturated with a liquid containing the additives is saved until transformation to the dry state.

5 A porous monolithic bed is typically introduced during the manufacture of the device, for instance

- a) by polymerization, or
- b) by placing ready-made porous monoliths

in each of at least a subset of the reaction microcavities of the microfluidic device.

10

In alternative a) the preferred variant is to carry out the polymerization with the reaction microcavity and the corresponding microchannel structure in an enclosed form. In alternative b) the preferred variant is to insert the monolith while at least the reaction microcavity is uncovered. After introduction of the porous bed and if needed

15 enclosing the microcavity, the beds are saturated with a solution comprising the additives discussed above and saved until transformation to the dry state.

Transformation of the beds to the dry state may be accomplished by removing the liquid under subatmospheric pressure, for instance below and/or above the freezing point of the liquid they are saturated with. Removal under subatmospheric pressure

20 and below the freezing point typically means lyophilization (lyophilization).

Alternatively liquid is removed from the settled dispersion under ambient atmosphere with or without warming. In the case the device is intended for using centrifugal force for driving liquid flow so called spin-drying may be employed. See the experimental

25 part.

The reconstitution of the wet porous beds means that a reconstitution liquid is allowed to flow through each of the reaction microcavities containing solid phase material in a dry state. See the experimental part.

30

An important tool for treating the solid phase material equal in several structures is to provide each microchannel structure with an inlet arrangement that in preferred variants is common to a group of microchannel structures/reaction microcavities as

GY 0068SE

24

2004-0129

M 100, II p. 10 Kazeem

discussed for the first aspect for parallel dispensation of solid phase material and reconstituting and conditioning of porous beds. In order to accomplish the best benefits of the invention it is thereby important to provide inner surfaces of at least the inlet arrangements, distribution manifold, and/or individual volume-metering units 5 with hydrophilic surface characteristics within the limits discussed elsewhere in this specification and the outlet of each volume-metering unit with a valve function that preferably is passive in the sense that it is without movable parts, for instance in the form of a local hydrophobic surface break.

#### 10 THIRD ASPECT OF THE INVENTION. THE USE OF THE DEVICE.

The use of the innovative microfluidic devices comprises in general terms the steps of:

- (i) providing a microfluidic device according to the first aspect of the invention;
- 15 (ii) reconstituting the solid phase material that is in the dry state to a wet porous bed in a predetermined number of the microchannel structures /reaction microcavities, preferably under flow conditions,
- (iii) providing a liquid containing a solute in a position that is upstream to said wet porous bed in one or more of the microchannel structures containing 20 the wet porous bed,
- (iv) transporting the liquid through said wet bed in at least one of said one or more microchannel structures.

Step (iii) comprises that the solute is formed within the device/microchannel structure 25 or is dispensed to the microchannel structure. If applicable, formation is typically in a position upstream or within the bed. Dispensing is typically to a position upstream the bed and via an inlet port.

The transport during step (iv) comprises that interaction between the immobilized 30 affinity counterpart AC<sub>S</sub> and the solute S may take place under static or flow conditions, i.e. with or without transport of the solute by a liquid flow passing through the reaction microcavities. We have previously found that more information may be gained under flow conditions (WO 0275312 (Gyros AB), for instance with the flow

GY 0068SE

25

101-29

2004-0129

rate and/or residence time adjusted to give conditions such that the distribution of bound solute in the solid phase will reflect the actual reaction rate or affinity between an immobilized affinity reactant, typically ACs, and a solute, typically solute S, with a minimum of perturbation by diffusion (non-diffusion limiting conditions). This also applies to the present invention but does not exclude that for applications where the primary interest is the total amount of bound/captured solute, capturing under flow conditions utilizing either diffusion limiting or non-diffusion limiting conditions can be used. The appropriate flow rate through the porous bed thus depends on a number of factors, e.g. the immobilized reactant and the solute and their sizes, the volume of the reaction microcavity, the porous bed including the solid phase material etc.

Typically the flow rate should give a residence time of  $\geq 0.010$  seconds such as  $\geq 0.050$  sec or  $\geq 0.1$  sec with an upper limit that typically is below 2 hours such as below 1 hour. Illustrative flow rates are within 0.01-1000 nl/sec, such as 0.01-100 nl/sec and more typically 0.1 - 10 nl/sec. These flow rate intervals may be useful for bed volumes in the range of 1-200 nl, such as 1-50 nl or 1-25 nl. Residence time refers to the time it takes for a liquid aliquot to be in contact with the solid phase in the reaction microcavity.

#### Steps (iii) and (iv)

20 These steps are performed in order to allow for an interaction between the solute and the porous bed to take place. As mentioned in the introductory part, the steps may be part of (a) separation method, and/or (b) a catalytic reaction, and/or (c) a solid phase synthesis.

25 Separation comprises among others:

- i) capturing, i.e. the porous bed exhibits an affinity structure (affinity ligand, affinity reactant) with binding ability for the solute such that when a liquid containing the solute passes through the bed then the liquid without solute or with a reduced amount of the solute will appear as a eluate, and/or
- 30 ii) size exclusion, i.e. the porous bed is more prone to retain smaller molecules compared to larger molecules, and/or
- iii) electrophoresis, i.e. the porous bed functions as anti-convection and/or anti-diffusion means, and/or

GY 0068SE

26

2004-01-29

2004-0129

iv) immobilization of the solute on the solid phase material for later use of the solid phase material so modified in capturing, catalytic reactions and solid phase synthesis.

- 5 For capturing (alternative i), the solute is adsorbed to the solid phase material and forms an immobilized affinity complex comprising the affinity structure and the solute. For many separation protocols a combination of capturing, size exclusion, electrophoresis etc is utilized.
- 10 For immobilization (alternative iv), the starting solid phase exhibits an affinity structure ( $AC_S = L'$ ) that is a counterpart to a conjugate ( $B' \cdot AC_S = S$ ) which has two kinds of binding sites: one ( $B'$ ) directed towards the affinity structure ( $L'$ ) on the solid phase and the other one towards a solute ( $S'$ ).  $L'$ ,  $B'$ ,  $S'$  and  $AC_S$  then correspond to  $L$ ,  $B$ ,  $S$  and  $AC_S$  and what has been said in the context of the first aspect for  $L$ ,  $B$ ,  $S$  and  $AC_S$  including affinity constants also applies here.

The separation may be part of a purification or enrichment protocol for a solute that is present in the liquid. The solute that is separated from the liquid may be a contaminant or the entity to be purified, enriched etc. The separation may also be part 20 of a synthetic protocol, preparative protocol, a cell based assay, various kinds of affinity assays including nucleic acid assays, immunoassays, enzyme assays etc.

An affinity assay utilizing a capturing step for binding a solute to a solid phase material typically contemplates characterization of a reaction variable involved in an 25 affinity reaction of the assay. Reaction variables in this context are mainly of two kinds 1) variables related to affinity reactants, and 2) reaction conditions. Variables related to affinity reactants have two main subgroups a) amounts including presence and/or absence, concentration, relative amounts, activity such as binding activity and enzyme activity, etc, and b) properties of affinity reactants including affinity as such, 30 e.g. affinity constants, specificities etc. See WO 0275312 (Gyros AB). The molecular entity for which a reaction variable of type 1 is characterized is called an analyte.

GY 0068SE

27

7 712 -01- 2 9

2004-0129

Catalytic reactions in the context of the present invention comprises that the solid phase material exhibits one or more immobilized members (affinity structure, affinity ligand, affinity reactant) of the catalytic system utilized, while other members of the same system are solutes. The catalytic reaction comprises formation of an affinity complex between the immobilized member (affinity structure, affinity ligand, affinity reactant) and at least one of the solute members. At least one of the members corresponds to the substrate for the catalytic system. The reaction results in a product that typically has a different chemical composition and/or structure compared to the substrate. The product may or may not become immobilized to the bed during the reaction.

The term "catalytic system" includes single catalytic system and more complex variants comprising a series of linked single enzyme systems, whole cells, cell parts exhibiting enzymatic activity etc. The bed may function as a catalytic reactor, such as an enzyme reactor.

The step during which interaction with the solute occurs may be part of a catalytic assay, such as an enzyme assay, for characterizing one or more members of the catalytic system or other reaction variable (e.g. reaction condition). The assay may be for determining the activity of a particular catalyst, substrate, co-substrate, cofactor, co-catalyst etc in a liquid sample. The molecular entity/entities corresponding to the activity to be determined is/are called analyte/analytes. See for instance SE 0201310 (Gyros AB).

In the context of assays, the term analyte includes the entity to be characterized in an original sample as well as analyte-derived entities formed during the assay and being related quantitatively and the analyte in the original sample. The solute discussed above may be the original analyte or an analyte-derived entity.

Solid phase synthesis includes for instance polymer synthesis, such as oligopeptide and oligonucleotide synthesis and synthesis of other small molecules on a solid phase material. The immobilized reactant in used in polymer synthesis, for instance, may exhibit the structure of the corresponding monomer, such as nucleotide, carbohydrate,

GY 0068SE

28

-01- 2 9

2004-0129  
amino acid structure, and mimetics of these structures. Synthesis of libraries of immobilized members of combinatorial libraries is also included. Such members have relatively low molecular weights (e.g. < 10,000 dalton including a possible spacer to a polymeric backbone).

5

**A MICROFLUIDIC DEVICE COMPRISING SEGMENTED POROUS BEDS (THE FOURTH ASPECT)**

A separate innovative aspect (4<sup>th</sup> aspect) is a microfluidic device that comprises one, two or more microchannel structures, each of which comprises a reaction microcavity 10 in which a solid phase material in the form of a wet porous bed is retained. A reactant is exposed in immobilized form on the solid phase material and capable of interacting with a solute (S) in a liquid passing through the bed.

During experiments with different kinds of samples when carrying out experiments as 15 outlined in this specification and in WO 0275312 (Gyros AB) applicant has found that samples many times may contain disturbing substances that are capable of negatively affecting results related to desired reactions between an immobilized reactant and a solute. These substances may be low molecular compounds, aggregates and/or particulate material. For biologically derived samples particulate material may be cell 20 debris and the like, lipids etc. The problem encountered may be linked to the type of solute that is to react with the immobilized reactant, thus for instance membrane associated biological molecules typically are accompanied by relatively large amounts of particulate material, solutes and the like that are difficult to handle in a microfluidic device, for instance in cell lysate preparations and other samples deriving from cells, 25 tissue and body fluids.

One of the present inventors has realized that these problems many times can be wholly or partially overcome by designing the porous bed to have an upstream part that is not exposing the immobilized reactant to a through-passing liquid containing 30 the solut (dummy or blank part in relation to solute (S). This inventor has also realized that by properly selecting the solid phase material in the upper part it will be possible to delay disturbing particulate material and/or disturbing low molecular weight solutes

GY 0068SE  
2004-0129  
to enter downstream into the microchannel structure where the desired reaction is to  
take place.

29

PLA 34-29

Other kinds of segmented porous beds in microfluidic devices have previously been  
5 suggested for other purposes. See US 6,632,655 (Caliper).

The characteristic feature of the 4<sup>th</sup> aspect is thus that the porous bed comprises a downstream part and an upstream part abutting the downstream part, possibly with a porous membrane between the parts securing physical separation of the material of  
10 one part from the material of the other part. The immobilized reactant is then primarily exposed in the downstream part.

According to this aspect the solid phase material in both parts may be in wet or in dry state as discussed for the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> aspects. The solid phase material may or may  
15 not contain a bed-preserving agent of the kind discussed for the same aspects.

This 4<sup>th</sup> aspect also comprises the use as defined for the 3<sup>rd</sup> aspect with the proviso that the microfluidic device provided in step (i) is according to this fourth aspect, and step (ii) is only needed in the case the solid phase material is in dry state.

20 The solid phase material in the upstream and the downstream part may be of the same or different kind. Thus the downstream part may be a porous monolithic plug and the upstream part a packed bed of particles, or the other way round, or both parts may be porous monoliths or porous beds of packed particles. The solid phase material in the  
25 two parts may also differ with respect to one or more of base material, particle size (and particle size distribution), porosity, coatings, hydrophilicity/hydrophobicity, swellability, elasticity, rigidity etc. Typically these features are selected as generally outlined for the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> aspects.

30 The upstream part of the porous bed typically acts as a filter for mechanically preventing particulate material in a sample from penetrating the downstream part in which the intended reaction is to take place.

GY 0068SE

30

2004-0129

The solid phase material is preferably a size exclusion material because this kind of media with the appropriate size exclusion at least potentially will render it difficult or even prevent a solute having a smaller size than

- 5      a) the solute S that is to react with the immobilized reactant, and/or  
      b) other reactants that are needed for desired reactions downstream the upper part of the porous bed

from penetrating the lower part of the porous bed. Preferred size exclusion material or media are liquid chromatography size exclusion material including in particular gel filtration material. In this context "a smaller size" typically refers to a lower molecular weight and/or a smaller hydrodynamic size. Low molecular weight solutes may be found amongst solutes having molecular weights  $\leq$  100,000 dalton, such as  $\leq$  50,000 dalton or  $\leq$  10,000 dalton or  $\leq$  7,000 dalton and are ultimately determined relative to the molecular weight and/or size of the solute(s) that one wants to pass through the upstream part.

15

It is believed that proper size exclusion media are selected amongst this kind of media that have Kav-values  $<$  0.5, such as  $<$  0.4 or  $<$  0.1, for solutes that are to pass through the upstream part of the porous bed and used as reactants downstream this part. More particularly suitable size exclusion media should have a Kav-value within the range of 20 0.1-0.95, typically within 0.40-0.95, for those solutes that are to be prevented from reaching the downstream part of the porous bed. For a definition of Kav see L. Hagel in "Protein Purification, Principles, High Resolution, and Applications", J-C Janson and L Rydén (Eds), VCH Publishers Inc. New York, 1989, p. 99.

25 Typical size exclusion media comprise particles that may be packed to a porous bed. Size exclusion media may also be in the form of monolithic porous plugs comprising through-passing pores in which convective mass transport can take place and other smaller pores in which primarily mass transport by diffusion takes place. See for instance WO9839094 (Pharmacia Biotech AB).

30

The immobilized reactant is typically selected amongst the same kinds of immobilized reactants as discussed for the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> aspects of the invention. Thus the reactant in the most typically embodiments of the invention is an affinity reactant (AC<sub>s</sub>) that is

GY 0068SE

31

2004-0129

an affinity counterpart ( $AC_1$ ) to the solute (S). Other reactants are also selected according to the outlining for these aspects.

- The solid phase material in the upstream part may also expose an immobilized reactant which typically binds to a different solute compared to the immobilized reactant in the downstream part. The immobilized reactant, if present, in the upstream part may be selected amongst the same groups as the reactant in the downstream part except that it has essentially no binding to the solute S when attached to the solid phase material.

10

Other embodiments given for the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> aspects are typically also applicable to the 4<sup>th</sup> aspect.

#### BEST MODE

- 15 The best mode of the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> aspects of the invention at the filing of this application is given in the experimental part and encompasses the solid phase materials shown, trehalose as bed-preserving agent, potassium phosphate as additional additive (buffer), and a microfluidic device with the microchannel structures given in figure 1.

20

The best mode of the 4<sup>th</sup> aspect is given in the experimental part.

#### EXPERIMENTAL PART

##### FIGURES

- 25 Figure 1 gives a subgroup of microchannel structures of the microfluidic device utilized in the experimental part.

- Figures 2a and b show a swellable solid phase material in particle form (Superdex Peptide, Amersham Biosciences, Uppsala, Sweden) placed in a reaction microcavity (104a-h). In figure 2a the particles have been lyophilized and lumped together scattered randomly in the reaction microcavity. No packed bed is at hand. In figure 2b the solid phase material has been reconstituted to well-ordered a porous bed.

30

GY 0068SE

32

7002 -01- 2 9

2004-0129

Figure 3 shows monodisperse essentially non-swellable and hydrophilic particles

Figur 3. Monodisperse Partiklar

packed to a porous bed after lyophilisation in a reaction microcavity (104a-h). The bed looked essentially the same after reconstitution (not shown) and therefore no figure has been included.

5 Figures 4a and b show the effect of drying (lyophilization) together with potassium phosphate buffer on the performance of a packed bed of particles to which streptavidin had been immobilized. Fluorescence intensity in radial direction through the bed is given. Storage for one month at +4°C. The effect is measured in a fluorescence myoglobin immunoassay at four different myoglobin concentrations and compared with the performance of a bed of the same material that has not been dried (lyophilized) (slurry). The myoglobin concentrations were 4.56 nM (1), 22.8 mM (2), 91.2 (3) and 273.6 (4). Figure 4a lyophilization and storage together with potassium phosphate. Figure 4b without lyophilization.

10 15 Figures 5a-d show the effect of three different drying procedures with a bed-preserving agent (sugar variant, trehalose) on the performance of a packed bed of particles to which streptavidin is covalently coupled. Storage and measurement is the same as for figures 4a-b. Figure 5a is without drying (slurry), figure 5b spin-drying, figure 5c is vacuum-dried, and figure 5d

20 lyophilization.

25 25 Figures 6. Integrated signals are shown for three antibody pairs with the different porous beds. In each group the left column represents the non-segmented bed. The middle and right column represent alternative (a) and (b), the middle column the alternative (a). The bars represent the total integrated signals from undiluted stimulated cell lysate prepared in triplicates. The segmented bed with the Superdex™ peptide revealed increased signals for all three antibody combinations.

30 30 The microfluidic device used for the experiments was circular and of the same dimension as a conventional CD (compact disc). The microfluidic device will be called CD in the experimental part. The CD contained 14 groups of 8 microchannel structures arranged in an annular zone around the center (spin axis) of the disc with a

Ink. t. Patent- och marken

GY 0068SE

33

2004-0129

1001-01-7-9

common waste channel for each group close to the periphery. A group of 8 microchannel structures is shown in figure 1 and is similar to and function in the

same manner as the group of microchannel structures illustrated in figures 1-2 of (WO

0275312, Gyros AB) and the corresponding figures in PCT/SE02/01678 (Gyros AB)

5 and PCT/SE02/01701 (Gyros AB). The dimensions are essentially of the same size as in these earlier patent applications.

Each subset (100) comprises 8 microchannel structures (101a-h), one common inlet arrangement (102) and eight separate inlet arrangements (103a-h), and eight reaction 10 microcavities (104a-h). The common inlet arrangement comprises a) two inlet ports (105a-b) that also will function as outlet port for excess liquid, and b) one volume-metering unit (106a-h) for each microchannel structure (101a-h). The volume-metering units (106a-h) will function as a distribution manifold for the downstream parts of the microchannel structures. Each of the separate inlet arrangements (103a-h) 15 is part of only one microchannel structure and comprises an inlet port (107a-h) and a volume-metering unit (108a-h). Between each volume-metering unit (106a-h, 108a-h) and their downstream parts, respectively, there is a valve function (109a-h, 110a-h), preferably passive. A reaction macrocavity (104a-h) of a microchannel structure (101a-h) is located downstream both the common inlet arrangement (102) and the 20 separate inlet arrangement (103a-h) of a microchannel structure (101a-h). At the outlet end (111a-h) of each reaction microcavity, the depth is lowered from 100 µm to 10 µm in two steps to prevent particles from escaping the reaction microcavity. At the periphery there is a common waste channel (112). Vents (113a-i) together with the valves (109a-h) define the volume of the liquid aliquots to be distributed downstream 25 from each the volume-metering unit (106a-h).

By applying the appropriate volume of aqueous liquid to the inlet port of an inlet arrangement, capillarity will fill the volume-metering unit(s) connected to the inlet port with liquid. By spinning the disc around its center, liquid can be forced to pass 30 the valve between a volume-metering unit and downstream parts.

## EXPERIMENTALS

### Instrumentation

GY 0068SE

34

2004-0129

The immunoassay was performed in an automated system. The system (Gyrolab Workstation, prototype 2 instrument equipped with a Laser Induced Fluorescence (LIF) module, Gyros AB, Uppsala, Sweden) was equipped with a CD-spinner, holder for microtiter plates (MTP) and a robotic arm with a holder for 10 capillaries

- 5 connected to 5 syringe pumps, 2 and 2. Two of the capillaries transferred all the reagents and buffers from a MTP to either of the two common inlet ports (105a-b) in the CD. The other eight capillaries transferred individual samples from a MTP to the separate individual inlet ports (107a-h) in the CD.
- 10 The Gyrolab Workstation is a fully automated robotic system controlled by application-specific software. An application specific method within the software controls the spinning of the CD at the precisely controlled speeds and thereby controls the movement of liquids through the microstructures as the application proceeds. Special software was included in order to reduce background noise.

15

See also WO 02075312 (Gyros AB), PCT/SE02/01678 and US 10/062,258 (Gyros AB), and PCT/SE02/02455 and 10/331,399 (Gyros AB) and also [www.gyros.com](http://www.gyros.com).

- Solid phase, immobilization of streptavidin, packing, drying/dehydration and  
20 reconstitution
- The solid phase bead material packed in the microstructures of the microfluidic device could be of either a porous or solid nature. For example polystyrene particles (15 mm, Dynal Biotech, Oslo, Norway) were selected for the solid phase. The beads were modified by passive adsorption of phenyl-dextran (Phe-Dex) to create a hydrophilic  
25 surface and were subsequently covalently coupled with streptavidin (Immunopure Streptavidin, Pierce, Perbio Science UK Limited, Cheshire, United Kingdom) using CDAP chemistry (Kohn & Wilchek, Biochem. Biophys. Res. Commun. 107 (1982), 878-884). Other particles as Superdex Peptide and Sepharose HP (Amersham Biosciences, Uppsala, Sweden) have also been covalently coupled with streptavidin  
30 using CDAP chemistry (without phenyl-dextran coating). Streptavidin-biotin is a well-known bioaffinity pair. The polystyrene particles are solid and non-swellable. Superdex Peptide and Sepharose HP are porous for many affinity reactants and swellable in the liquids used.

GY 0068SE  
2004-0129

35

Ink. t. Patient- och rap verket

711-01-2-9

Hans Rydberg, MSc

After coupling with the streptavidin, a suspension of the particles in potassium phosphate buffer (10mM) without bed-preserving agent or with bed-preserving agent (in this case a sugar additive (10-100 mM)) was distributed in the common

5 distribution channel via inlet port (105a-b) and moved through the structure by centrifugal force. The centrifugal force combined with the vents (109a-h,113a-i) divide the suspension in the common inlet arrangement (102) in equal portions, each of which forms a bed of packed particles (column) in each reaction microcavity (104a-h) against the dual depth (111a-h). The approximate volume of the column was

10- 15 nl. The columns/beds were dried/dehydrated by three various methods:

Spin-drying: The microfluidic device containing the wet porous beds was spun for one minute at 6000 rpm to remove as much of the fluid as possible before the device was put into a jewel case and sealed in a polymer-coated aluminum bag.

Vacuum-drying: The microfluidic device containing the wet porous beds was placed 15 on trays and put into a vacuum drying oven (Heraeus vacutherm VT6060M). The temperature was set to 25 °C and the pressure was reduced by vacuum to 0.1 millibars. The device was maintained at this pressure and temperature for half an hours, until the product was dried. The pressure was then allowed to reach atmospheric pressure. The device was then placed into a jewel case and sealed in a 20 polymer-coated aluminum bag.

Freeze-drying (lyophilization): The microfluidic device containing the wet porous beds were placed on a tray and put into a -80 °C freezer. (The device could also be placed in an ordinary -20 °C freezer for an hour.) After a few minutes all columns in the device were freezed and the trays where transferred to a freeze-dryer apparatus (Heto, 25 LyoPro 3000) in which the condenser temperature was set to -57 °C. The pressure was reduced (by vacuum) to 0.1-0.06 millibars. The device was maintained at this pressure and temperature until all of the ice had sublimed (about 12 hours or over night). The pressure was then allowed to reach atmospheric pressure during 2 minutes before the chamber was opened and the lyophilized product was provided in the 30 device. The device was put into a jewel case and sealed in a in a polymer-coated aluminum bag.

GY 0068SE  
2004-0129

36

-01-29

Hans-Joachim Hartmann

The devices were stored for one month at +4°C after which the dry columns were rewetted/reconstituted once with 15 mM phosphate buffer (PBS), pH 7,4 containing 0,15 M NaCl, 0,02% NaN<sub>3</sub> and 0,01% Tween 20 via the common distribution channel and spinning at the appropriate rate. Every addition of solution delivers 200 nl liquid

5 to the individual column (104a-h). Finally the function of the reconstituted beds was tested in the immunoassay given below at four different analyte (myoglobin) concentrations and compared with the corresponding beds that had not been dried/dehydrated. The results are presented in figures 4-5 and show that it is imperative to include a bed-preserving agent in order to reconstitute the

10 dry/dehydrated solid phase material to an efficient wet porous bed.

### Immunoassay

The catching antibody in our myoglobin assay, the monoclonal antimyoglobin 8E11.1 (LabAS, Tartu, Estonia) was biotinylated using Sulfo-NHS-LC-biotin (Pierce, prod # 15 21335, Perbio Science UK Limited, Cheshire, United Kingdom). The protein concentration of the monoclonal antimyoglobin 8E11.1 was 1-10 mg/ml and it was incubated in room temperature for 1 h with 3× molar excess of the biotinylation reagent in 15 mM PBS with 0,15 M NaCl before it was gel filtrated through either a NAP-5 column (Amersham Biosciences, Uppsala, Sweden) or a Protein Desalting

20 Spin Column (Pierce, # 89849-P, Perbio Science UK Limited, Cheshire, United Kingdom).

To load the streptavidin immobilized particles with the biotinylated antibody, a solution at a 0,2-2 mg/ml concentration (depending of how much streptavidin it is in 25 the packed column) of antibody was distributed in the common distribution channel via inlet port (105a-b) and moved through the structure by centrifugal force. The flow rate through the columns was controlled by the spin velocity (spin flow 1). After the capturing antibody was attached to the columns they were washed once by addition of PBS (with 0,01% Tween 20) to the common distribution channel (inlet ports 105a or 30 b) followed by a spin step.

To demonstrate the myoglobin assay in Gyrolab Workstation a 6-point standard curve was created. The myoglobin samples (diluted in PBS with 1% BSA) with

GY 0068SE

37

2004-0129

concentrations in the range of 0-274 nM were distributed to the individual inlet ports (107a-h) by the capillaries. The sample volume 200 nl was defined into the volumemetering unit (108a-h), during the first two steps in the spin flow method. To reach favourable kinetic condition under the capturing step (for myoglobin to bind to

- 5 8E11.1) the flow rate of the sample should not exceed 1 nl/sec. The sample flow rate was controlled by spin flow 2. After sample capturing the columns was washed twice by addition PBS (with 0,01% Tween 20) to the common distribution channel (inlet port 105a or b) followed by a spin step. Detecting antibodies (monoclonal antimyoglobin 2F9.1 (LabAs, Tartu, Estonia)) in excess were applied next via the
- 10 common distribution channel (inlet port 105a or b) and a similar slow flow rate (spin flow 3) was used. The detecting antibodies were labeled with a fluorophore Alexa 633 (Molecular Probes, Eugene, USA). Excess of labeled antibody was washed away by 4 additions of PBS (with 0,01% Tween 20) to the common distribution channel (inlet port 105a or b) followed by a spin step.

15

The complete assay was analyzed in the Laser Induced Fluorescence (LIF) detector module. See more WO 02075312 (Gyros AB), PCT/SE02/01678 and US 10/062,258 (Gyros AB), and PCT/SE02/02455 and 10/331,399 (Gyros AB).

- 20 An overview of the run method performed in the system is presented in Table 1.

Table 1

METHOD	SPIN PROFILE
Rewetting of bead columns	
Spin 1	2500 rpm 5s, 6000 rpm 10s
Wash of beads	
Spin 2	1200 rpm 2s, 2500 rpm 0,5s, 4000 rpm 10s, 6500 rpm 16s
Transfer of biotinylated antibody	
Spin flow 1	1200 rpm 2s, 2500 rpm 0,5s, from 1200-1500 rpm 45s, 2000 rpm 35s, 3000 rpm

29-01-2004 11:32

FRAN-GYROS AB

+46 18 566350

+46 18 566350

T-026 P.043/058 F-406

Ink t. Patient- och reg.verket

GY 0068SE  
2004-0129

38

1111-01-29

	30s, 4000 rpm 10s, 5000 rpm 5s, 6000 rpm 10s
Wash of beads and CD-structure 1	
Spin 3	1200 rpm 2s, 2500 rpm 1s, 4000 rpm 15s, 6000 rpm 18s
Transfer of myoglobin samples	
Spin flow 2	1000 rpm 5s, 2500 rpm 0,5s, from 1200-1500 rpm 90s, 2000 rpm 70s, 3000 rpm 60s, 4000 rpm 20s, 5000 rpm 10s
Myoglobin wash 1	
Spin 4	1200 rpm 2s, 2500 rpm 0,5s, 4000 rpm 10s, 6000 rpm 16s
Myoglobin wash 2	
Spin 5	1200 rpm 2s, 2500 rpm 0,5s, 4000 rpm 10s, 6000 rpm 16s
Transfer of conjugate	
Spin flow 3	1200 rpm 2s, 2500 rpm 0,5s, from 1200-1500 rpm 90s, 2000 rpm 70s, 3000 rpm 60s, 4000 rpm 20s, 5000 rpm 10s
Conjugate wash 1	
Spin 6	1200 rpm 2s, 2500 rpm 0,5s, 4000 rpm 10s, 6000 rpm 16s
Conjugate wash 2	
Spin 7	1200 rpm 2s, 2500 rpm 0,5s, 4000 rpm 10s, 6000 rpm 16s
Conjugate wash 3	
Spin 8	1200 rpm 2s, 2500 rpm 0,5s, 4000 rpm 10s, 6000 rpm 16s
Conjugate wash 4	
Spin 9	1200 rpm 2s, 2500 rpm 0,5s, 4000 rpm 10s, 6000 rpm 16s
Detection	

GY 0068SE  
2004-0129

39

2004-01-29

Referatnummer:

**Drying and reconstitution****COMPARISON BETWEEN SEGMENTED POROUS BEDS AND NON-SEGMENTED POROUS****5 BEDS**

This example is part of an investigation in which PDGF  $\beta$ -receptor in cell lysates from porcine aorta endothelial (PAE) cells stably expressing the membrane bound PDGF  $\beta$ -receptor and stimulated with +/- ligand PDGF-BB was assayed. The assaying method was a sandwich immunoassay utilizing one antibody raised against 10 the target protein and another antibody raised against one regulatory phosphorylated aminoacid or site. The sandwich immunoassay utilized the same streptavidin-phenyldextran coated solid phase as for assaying myoglobin above. The capture antibody was biotinylated by the same method as the capture anti-myoglobin antibody above. The detection antibody was labelled with Alexa 647 detection reagent by the 15 same methodology as the detection anti-myoglobin antibody above.

The assay was performed in the same kind of microchannel structure/microfluidic device/instrument as the myoglobin assay.

**20 Cell culture**

The cell lysate preparations derived from porcine aorta endothelial (PAE) cells stably expressing the PDGF  $\beta$ -receptor. The PAE cells were transfected with PDGF  $\beta$ -receptor and one of the cell cultures were also G-418 selected [Claesson-Welsh, L. et al. *cDNA cloning and expression of a human platelet-derived growth factor receptor specific for B-chain containing PDGF molecules*. Mol. Cell. Biol. 8 (1988) 3476-3486]. PAE cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum, 100  $\mu$ g/ml streptomycin and the amino acid glutamine. A nearly confluent monolayer of cells were starved overnight in Ham's F-12 supplemented with 0.1 mg/ml bovine serum albumin (BSA) and +/- stimulated with 100 ng/ml PDGF-BB for 30 60 minutes on a shake plater. Unstimulated cells are used as a control since they have no activated PDGF  $\beta$ -receptors, only unphosphorylated PDGF  $\beta$ -receptors. The receptors are activated and saturated with high concentration of ligand inhibiting internalisation followed by degradation during the stimulation. After the stimulation,

29-01-2004 11:32

FRAN-GYROS AB

+46 18 566350

+46 18 566350

T-025 Inkjet/EPSON dot matrix printer

2004-01-29

GY 0068SE

40

Huvudfunktionen

2004-0129

cells were washed two times in ice-cold PBS buffer and scraped off with a "rubber police men" in 1 ml PBS. The cell suspension were saved and lysed in 200 µl ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% Deoxycholate, 0.5 mM Na<sub>3</sub>VO<sub>4</sub> and 1% Trasylol from Bayer) for 15 min

- 5 on ice. The lysates were centrifuged at 13000 rpm for 15 min at 4°C and the supernatant were saved in aliquots and stored at -20°C. The cell lysate preparation was done twice and the first time the cells were grown in 75 cm<sup>2</sup> culture dishes and the second time they were grown in 175 cm<sup>2</sup> culture flasks in order to make more concentrated lysates. The quantitation of total protein was done according to BCA
- 10 Protein Assay Kit Microplate procedure from PIERCE Biotechnology (Boule Nordic AB, Huddinge, S.) and this Protein assay is based on bicinchoninic acid (BCA).

#### *PDGF β-receptor antibodies*

- The rabbit polyclonal antibody 958 directed against a recombinant protein corresponding to amino acids 958-1106 of the carboxy terminus of human PDGF β-receptor, the goat polyclonal antibody P-20, raised against a peptide of the carboxy terminal of human PDGF β-receptor, and the mouse monoclonal PY99 were all from Santa Cruz Biotechnologies (Santa Cruz, CA). The PDGF β-receptor antibodies P-20 and 958 are recommended for the detection of PDGF receptor type β of human and, to a lesser extent, mouse and rat origin by western blotting, immunoprecipitation and immunohistochemistry and should not be cross reactive with PDGF receptor type α. The antibodies have been used extensively in immunoprecipitaion and Western Blot experiments with the same cell culture, one example described in reference [Pietras K., et al. Inhibition of PDGF Receptor Signaling in Tumor Stroma Enhances Antitumor Effect of Chemotherapy. Cancer Research 62 (2002) 5476-548].

#### *Sandwich-based immunoassay methods*

- In the cell lysate assay, wash buffers and antibodies were distributed through the common distribution channel and cell lysate preparations were distributed through the individual inlet. Every batch run included standards in triplicates and blank samples; The cell lysate assay included a small dilution serie (+/- PDGF-BB) and several

GY 0068SE

41

H:\proj\T-025\059\req\verket

2004-0129

blanks with lysis buffer. All steps in the assay were automatically run in the Gyrolab Workstation.

- In the cell lysate assay several different combinations of the three antibodies were tested in order to find out what antibody pair that gives the highest degree of specific binding, 958/PY99, 958/P-20, P-20/P-20, P-20/958 and P-20/PY99 (capturing/detecting).

- By titration of the detection antibody it was determined that the concentration of the detection antibody should be 400 nM.

The  $\beta$ -PAE cell lysate with +/- PDGF-BB stimulation was diluted in lysis buffer (2 x, 4 x, 8 x) and undiluted cell lysate was also included as a standard point.

- 15 *Immobilization of capture antibody:* The porous beds were washed twice with wash buffer PBS-T (0.015 M Na<sub>2</sub>PO<sub>4</sub> pH 7.4, 0.15 M NaCl, 0.01% NaN<sub>3</sub>, 0.01% Tween-20) in order to recondition the streptavidin-coated particles followed by a short spin. The second step was addition of biotinylated capture antibody at a concentration of 667 nM followed by a spin and through the streptavidin-biotin interaction the capture antibody was bound to the solid phase. The capture antibody was diluted in wash buffer described above. After the immobilisation of capture antibody, two wash steps were performed with wash buffer and the same short spin as the previous wash steps.

- 25 *Basic assay protocol:* The cell lysate samples were added to the individual inlet in the microstructures. The subsequent spin made volume definition possible and 200 nl aliquots of sample were run into the porous beds, allowing capture antibody to interact with PDGF  $\beta$ -receptor molecules (analyte). Two wash steps were performed and the latter followed by a very short spin to ensure that the beds were filled with liquid during the fluorescence detection. The background fluorescence detection 30 included three detection steps with different sensitivity set on the LIF detector (laser induced fluorescence detector), 1%, 5% and 25%. Excess buffer was washed away by a short spin before the addition of the labelled detection antibody. The detection antibody was allowed to bind to the analyte during the following spin step and the

GY 0068SE

42

2004-0129

1997-01-01 Kungsan

sandwich immunoassay was formed with capture antibody on solid phase, analyte attached to capture antibody and detection antibody bound to analyte. Six wash steps were included, two times with normal wash buffer and four times with wash buffer including isopropanol 20%, to remove all unreacted detection antibody.

5

- In the beginning cell lysate runs were mainly performed according to the basic assay protocol with the capture anti body being distributed all over the porous bed. Modifications were tested because of difficulties to establish a specific interaction between the PDGF $\beta$ -receptor and the antibodies. First of all, the analyte and the 10 detection antibody spin program were extended and the linear flow over the bed reduced to enable the PDGF  $\beta$ -receptor in the cell lysate to attach to the capture antibody. After analyte addition three extra wash steps were included with PBS buffer without Tween-20 and the second and the fourth wash step were followed by a pulsed spin program, allowing the solution to move through the beds by simple diffusion. 15 The final wash steps after detection antibody was the same with the exception that isopropanol was excluded in the wash buffer.

- Due to a lot of uncontrolled non-specific interactions in the cell lysate assay a segmented porous bed containing an upstream part devoid of capture anti-analyte 20 antibody and a downstream part exposing anti-analyte antibody was loaded in reaction microcavities of the microstructures. The gel filtration particle Superdex<sup>TM</sup> peptide (Amersham Biosciences, Uppsala, S.) was loaded in a 5 x dilution slurry onto the existing polystyrene phenyldextran particle beds. Superdex<sup>TM</sup> peptide is used for high-resolution gel filtration of peptides and other small biomolecules of 100-7000 25 molecular weight.

## RESULTS

From pre-experiments three antibody pairs were selected P-20/958, P-20/PY99 and 958/PY99.

30

Non-segmented porous bed. In general the cell lysate runs revealed high CV values due to the signal distribution where a lot of variation between the triplicates was seen. In some cases a small difference in signal could be seen between undiluted cell lysate

GY 0068SE

43

2004-0129

and blank signals but mostly significant signals were hard to distinguish from background signals. The enrichment in the beds was mostly irregular and a general bed pattern could not be seen, in some cases the signal had a tendency to progress down the porous bed.

5

*Segmented porous bed.* Two types of segmented porous beds were tested:

- a) a bed of polystyrene particles (essentially non-porous) coated with phenyldextran on top of a bed of polystyrene particles coated with phenyldextran to which streptavidin has been immobilized, and
- 10 b) a bed of Superdex™ peptide on top of a bed of polystyrene particles coated with phenyldextran to which streptavidin has been immobilized.

In particular for the Superdex™ peptide segmented bed showed an improvement in signals for the three antibody pairs compared to the non-segmented bed. See in figure 6. Differences between cell lysate and blank responses could also be discerned.

15

- Certain innovative aspects of the invention are defined in more detail in the appending claims. Although the present invention and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the invention as defined by the appended claims. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, composition of matter, means, methods and steps described in the specification. As one of ordinary skill in the art will readily appreciate from the disclosure of the present invention, processes, machines, manufacture, compositions of matter, means, methods, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized according to the present invention. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, means, methods, or steps.

GY 0068SE  
2004-0129

44

S1- Z 9

## CLAIMS

1. A microfluidic device that comprises one, two or more microchannel structures, each of which comprises a reaction microcavity retaining a solid phase material in the form of a wet porous bed, a reactant being exposed in immobilized form on the solid phase material and capable of interacting with a solute (S) in a liquid passing through the bed, characterized in that
  - a) the porous bed comprises a downstream part and an upstream part abutting the downstream part, possibly with a porous membrane between the parts securing physical separation of the material of one part from the material of the other part, and
  - b) the immobilized reactant is primarily exposed in the downstream part.
2. The microfluidic device according to claim 1, characterized in that the porous bed is a packed bed of particles in at least one of the upstream and the downstream part and a porous monolithic plug in the other part, if any.
3. The microfluidic device according to any of claims 1-2, characterized in that the solid phase material in the upstream part comprises a size exclusion material permitting the solute s to pass through essentially unhindered compared to one or more solutes that negatively affects desired reactions downstream the upstream part, said size exclusion material preferably being a liquid chromatographic size exclusion material such as a gel filtration material
4. A microfluidic device comprising one, two or more microchannel structures, each of which comprises a reaction microcavity intended for retaining a solid phase material in the form of a wet porous bed, characterized in that each of said one, two or more microchannel structures comprises the solid phase material in a dry state that also comprises a bed-preserving agent comprising one or more compounds having bed-preserving activity.
5. The microfluidic device according to claims 4, characterized in that at least one of said one or more compounds a) exhibit a hydrophilic group that may or may not be non-ionic, and b) are water-soluble.

29-01-2004 11:33

FRÄN-GYROS AB

+46 18 566350

+46 18 566350

T-025 P.050/058 F-406

Ink. t. Patent- och reg. verket

GY 0068SE  
2004-0129

45

2004-01-29

Huvudlinje Koden

6. The microfluidic device according to any of claims 4-5, characterized in that at least one of said one or more compounds is a polyol.
- 5 7. The microfluidic device according to any of claims 4-6, characterized in that at least one of said one or more compounds exhibits carbohydrate structure, such as polysaccharide structure or oligosaccharide structure.
8. The microfluidic device according to any of claims 4-7, characterized in that at least one of said one or more compounds is a disaccharide, preferably trehalose.
- 10 9. The microfluidic device according to any of claims 4-8, characterized in that at least one of said one or more compounds can be in a glassy state in at least a part of the interval +15-35°C.
- 15 10. The microfluidic device of any of claims 4-9, characterized in that at least one of said compounds is a microcavity adherence agent.

GY 006SE  
2004-0129

46

Ink t. Patent- och registreringsverket

19 01 2004

Helsingborg, Sweden

**A B S T R A C T**

A microfluidic device that comprises one, two or more microchannel structures, each of which comprises a reaction microcavity retaining a solid phase material in the form 5 of a wet porous bed, a reactant being exposed in immobilized form on the solid phase material and capable of interacting with a solute (S) in a liquid passing through the bed. The device is characterized in that

- a) the porous bed comprises a downstream part and an upstream part abutting the downstream part, possibly with a porous membrane between the parts 10 securing physical separation of the material of one part from the material of the other part, and
- b) the immobilized reactant is primarily exposed in the downstream part.

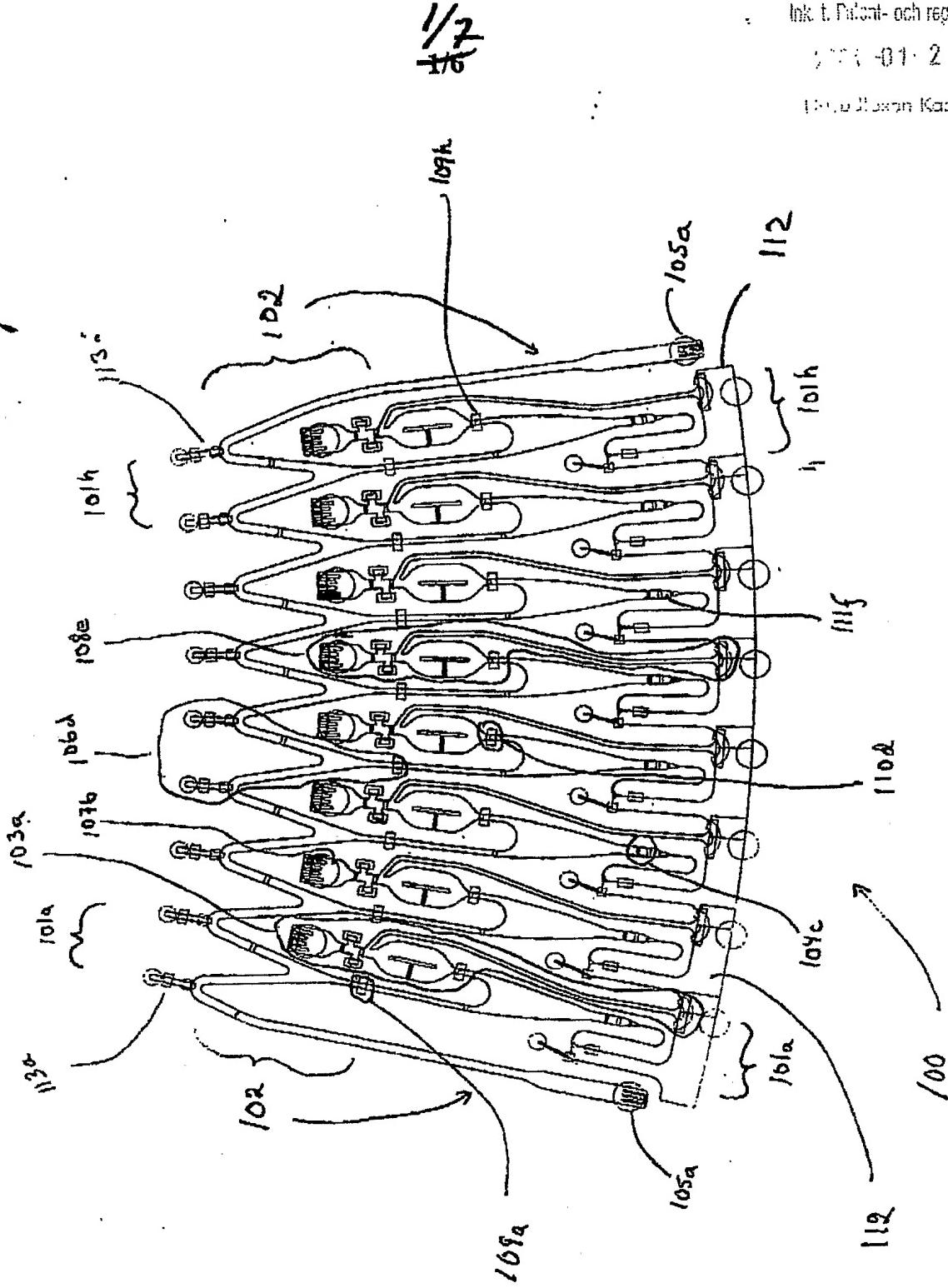
A microfluidic device comprising one, two or more microchannel structures, each of 15 which comprises a reaction microcavity intended for retaining a solid phase material in the form of a wet porous bed. Each of said one, two or more microchannel structures comprises the solid phase material in a dry state together with a bed-preserving agent comprising one or more compounds having bed-preserving activity.

Ink. t. Förbund- och reg.verket

2021-01-29

Die Japan-Kasse

1.  
Fig



29-01-2004 11:33

FRAN-GYROS AB

+46 18 566360

+46 18 566360

T-025 P.053/058 F-406

Lik t Patent- och registreringsverket

177-1-03-29

Vissa delar är saknade

2/2  
2/6

Figure 2a

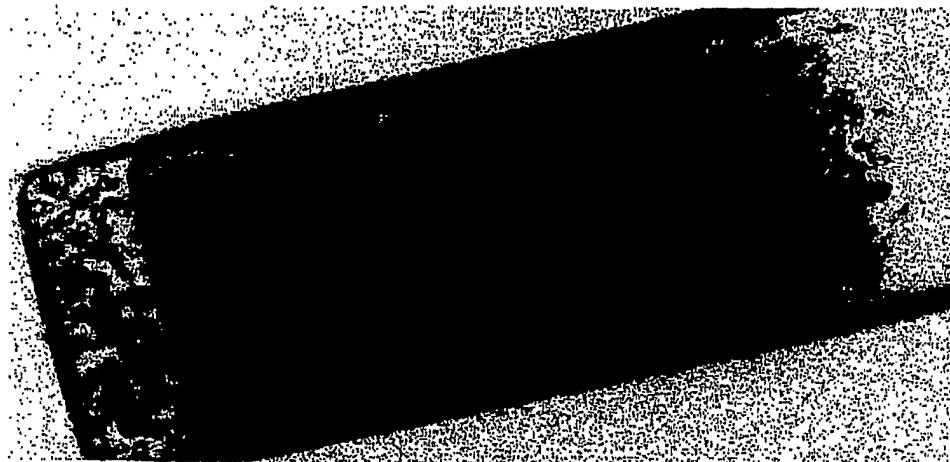
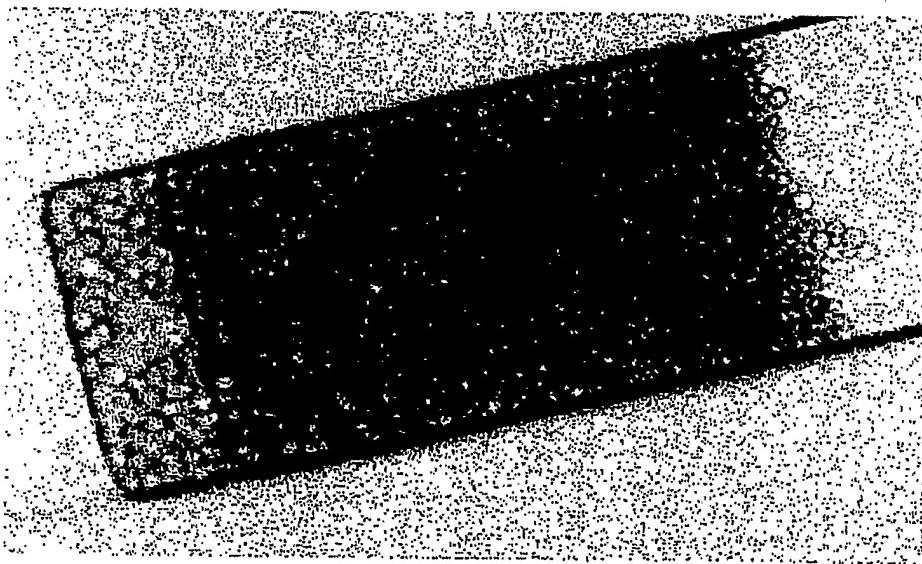


Figure 5b.



1  
2  
3  
4  
5  
6  
7  
8  
9

29-01-2004 11:39

FRÄN-GYROS AB

+46 18 566350

+46 18 566350

T-025 P.054/058 F-406

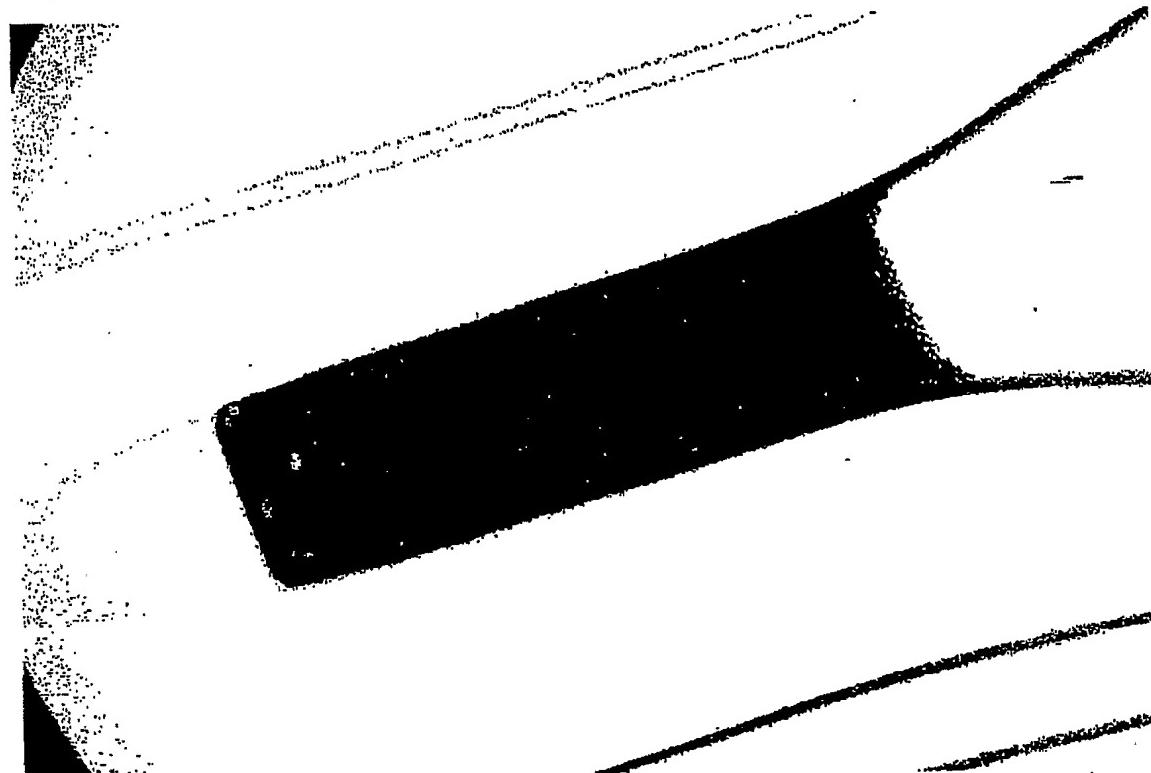
Jur. t. Patent- och reg.verket

29-01-29

Lit. och Ann. Kassan

3/6 3/2

Figure 3



29-01-2004 11:34

FRAN-GYROS AB

+4618566350

+46 18 566350

T-025 P.055/058 F-408

lk 1 Detalj-och reporvet

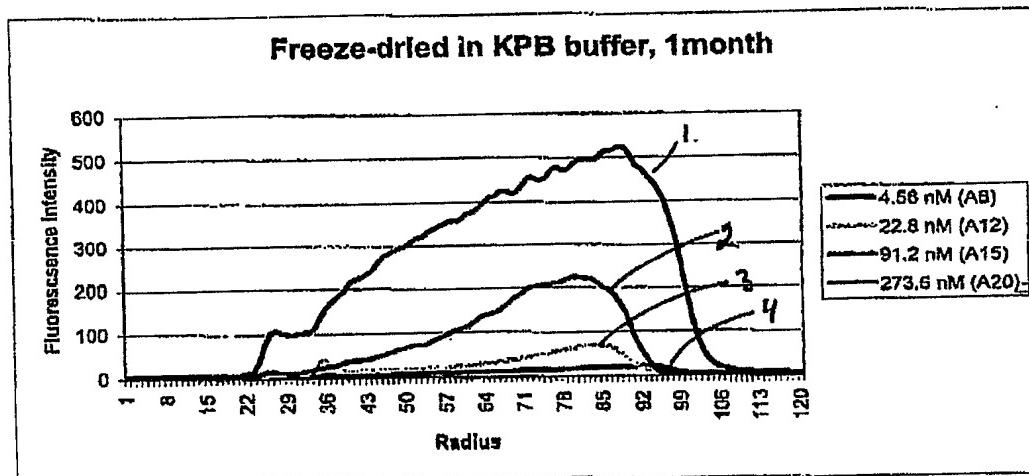
418

4/2

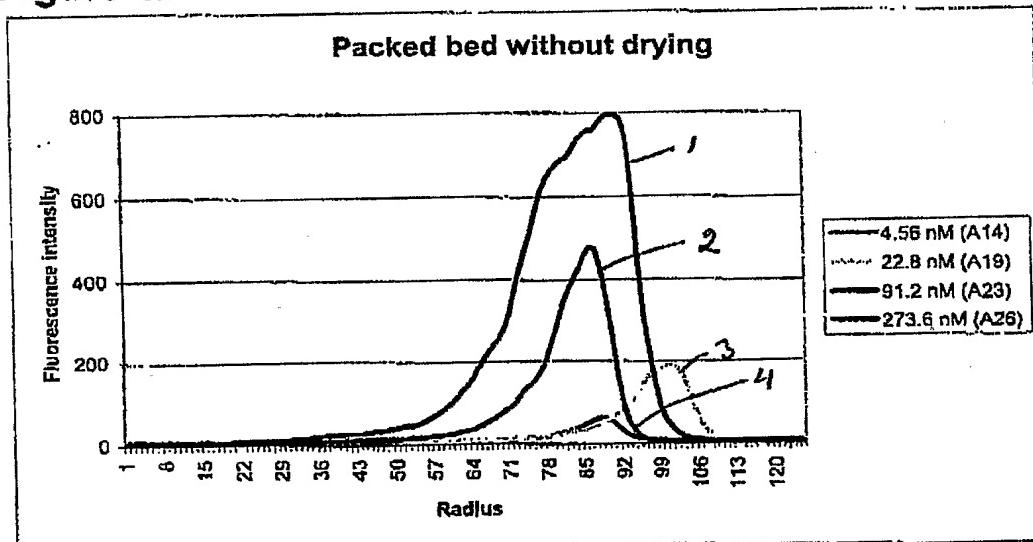
11-01-29

Frans Gyros AB

**Figure 4a**



**Figure 4b**



0.4 0.5 0.6 0.7 0.8 0.9 1.0

lok.t. Falun- och papper

C 1 - 57 - 9

Märk: 11.01.2004

5/6 5/2

Figure 5a

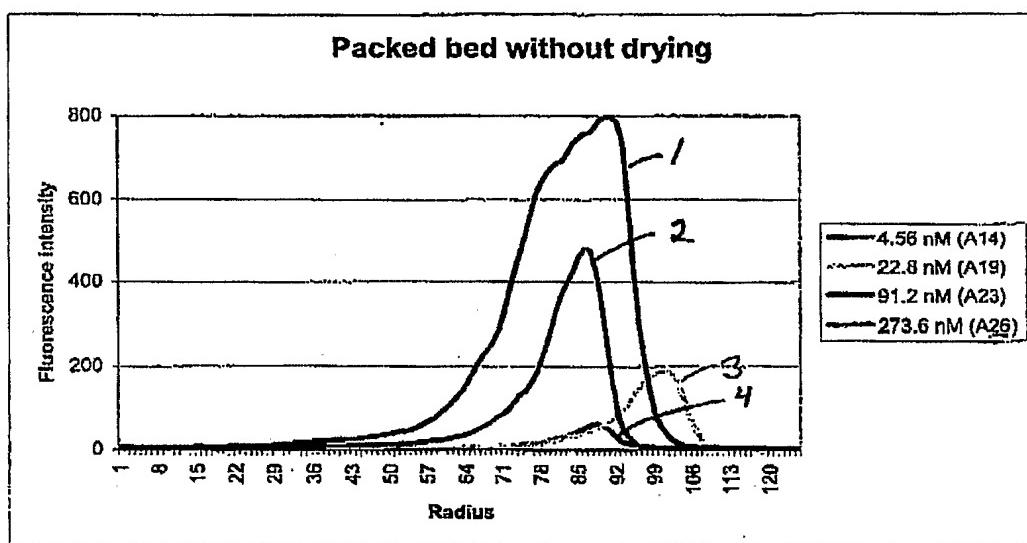
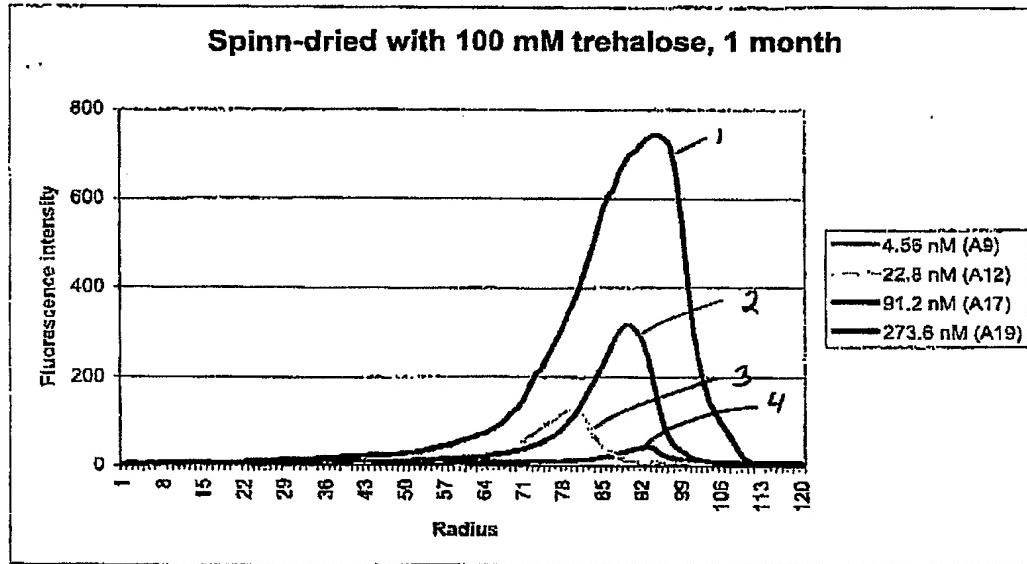


Figure 5b



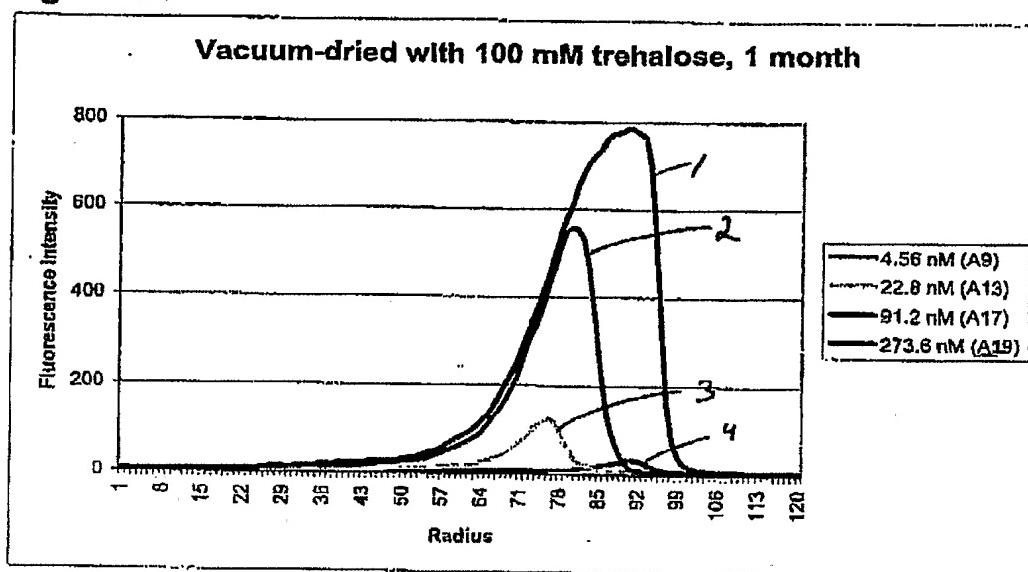
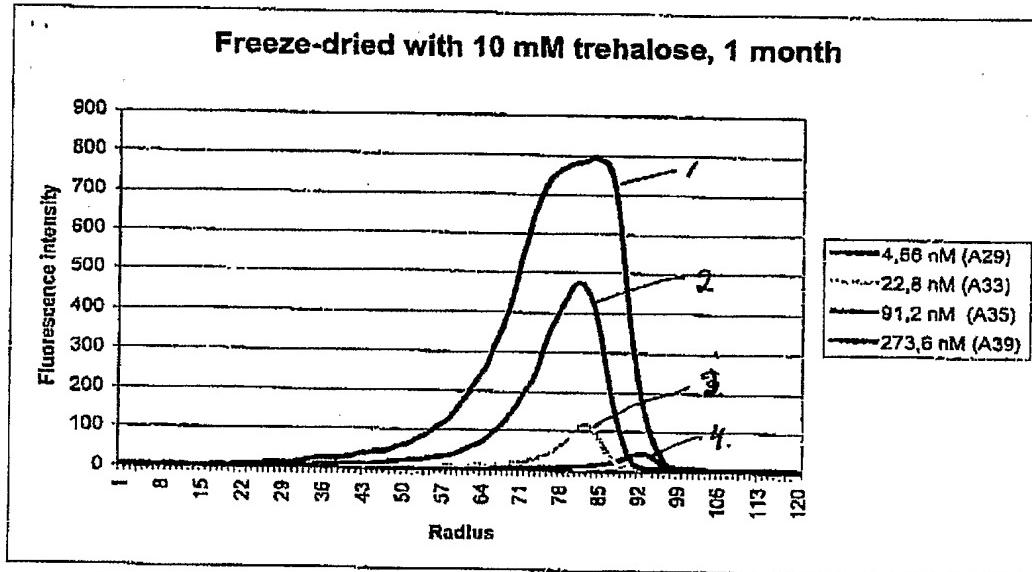
+4618566350

Ink t Patient- och renoverket

11-01-2004

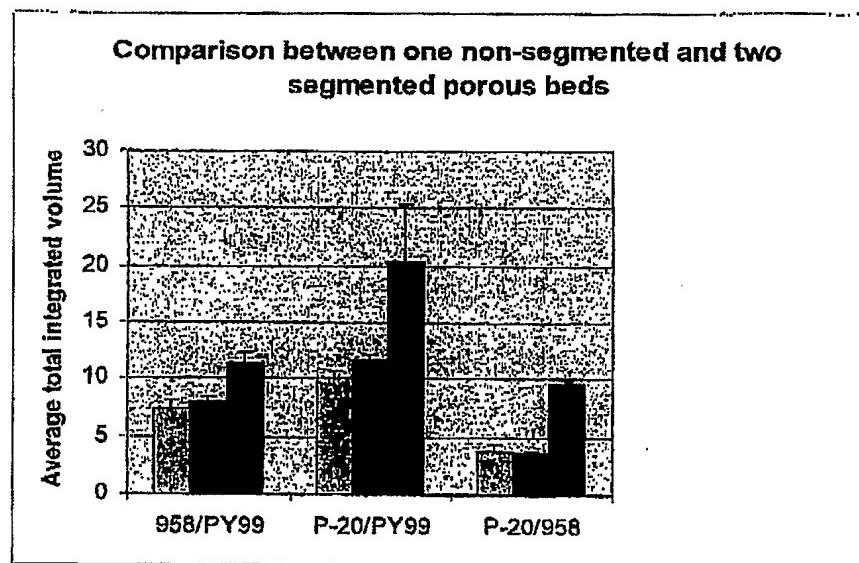
Player 1, 11.01.2004

246 6/2

**Figure 5c****Figure 5d**

77

Hauskunst-Karten



**Figure 6**